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13. ABSTRACT ***(Maximum 200 Words)***

Ovarian cancer is a highly lethal malignancy specific to women. We have setup the infrastructure at Mayo for an Ovarian Cancer Research Program utilizing the rich resources of clinical material and linking work from molecular geneticists with that of dedicated clinicians. We are proposing to combine several powerful strategies to clone many of the genes involved in ovarian cancer development. The first project focuses on identifying genes that are over-expressed or under-expressed during the development of ovarian cancer using subtraction suppression hybridization cDNA libraries and Gene Expression Array Analysis in collaboration with Millennium PredictiveMedicine.. The second project focuses on the role of gene amplification in familial versus sporadic ovarian cancer. The third project is to characterize two common fragile sites, FRA6F (6q21) and FRA6E (6q26) which are derived from chromosomal regions frequently deleted in ovarian cancer and that also contain genes involved in replication senescence. Thus we have three interactive projects whose overall focus is to identify key genetic targets in the development of ovarian cancer. This Program Project is centered within a larger institutional effort to better understand the biology of the development of ovarian cancer and to devise better strategies for prevention, early detection and treatment of this lethal disease.

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Introduction

Of the cancers unique to women, ovarian cancer has the highest mortality rate. Very little is known about the genetic alterations that result in the development of this lethal disease. However, it is clear that there may be many genetic changes that occur which lead to this disease. The purpose of this Program Project is to identify large numbers of ovarian cancer-related genes using the strategy of transcriptional profiling to identify large numbers of aberrantly regulated genes in primary ovarian tumors. We have been using microarrays containing 25,000 human genes to monitor the level of expression of these genes in ovarian tumors as compared to normal ovarian epithelial cells. This work will also be complemented with the construction of subtraction suppression hybridization cDNA libraries to identify additional aberrantly regulated genes, not on the microarrays. There are then three interconnected projects that will analyze some of the aberrantly regulated genes identified using transcriptional profiling. The first projects goal is to characterize the down-regulated genes and to test these as candidate tumor suppressor genes. The second project is to analyze over-expressed genes in both sporadic and hereditary ovarian cancer to characterize regions of amplification in sporadic versus hereditary ovarian tumors. The third project is to study the expression of genes that are localized to regions containing common fragile sites. The overall goal of this work is to better characterize the key genetic alterations that lead to the development of ovarian cancer. Although the work described in this Program Project was approved for funding over a year ago, we did not receive final approval for our proposed work with Human Subjects until the beginning of August. Thus, we are actually only describing work that was done over a several month period.

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PROJECT #1: Down-regulated genes in ovarian cancer. Viji Shridhar, P.I.

The specific goal of this project is to identify genes that are down regulated during the development of ovarian cancer with an expression based screening strategy. The two main strategies are: 1) Generate subtraction suppression cDNA libraries (SSH cDNA libraries between ovarian tumors from patients with different stages of disease and normal ovarian epithelial cells; and 2) In collaboration with Millennium Predictive Medicine (MPMx, Cambridge, MA), use their 25K gene expression arrays to screen for changes in expression of primary ovarian tumors of serous histology of different stages.

Genes identified from this preliminary screening will be characterized in the following ways:

(A) The expression profiles of down-regulated genes will be confirmed by Northern and semi-quantitative RT-PCR analysis in both in primary tumors and cell lines.

(B) The down-regulated genes from (A) will be analyzed on a corresponding Southern blot of DNA from primary ovarian tumors and cell lines to identify any altered or deleted bands at the genomic DNA level.

(C) Identify corresponding genomic BAC clones and map it to specific chromosomal regions either by FISH or by radiation hybrid mapping panel.

(D) Test candidate down regulated genes for mutations using the high throughput capabilities of denaturing high performance liquid chromatography.

(E) The final specific aim of this Project is to correlate the expression of down-regulated genes in a significant proportion of a large panel of primary ovarian tumors from patients for whom we have extensive outcome data. This will allow us to determine the clinical significance of alterations in these genes.

Research Accomplishments

We have used the microarrays containing 25,000 genes to analyze a total of 20 primary ovarian tumors. This included 10 early-stage tumors of different histologies and 10 late-stage serous ovarian tumors. This analysis revealed that there were more down-regulated than up-regulated genes in the tumors profiled. The Table below shows the number of down-regulated and up-regulated genes identified by this work in various categories depending upon how much each gene was up- or down-regulated and in what proportion of the tumors analyzed this was observed (out of the 20 analyzed). It is clear from these results that there are indeed a large number of aberrantly regulated genes in the ovarian tumor samples.

	Stage I DOWN					Stage III DOWN				
# of Tumors	2	3	5	10	20	2	3	5	10	20
10	119	53	30	9	1	250	94	46	18	6
9	255	101	46	14	6	480	172	69	21	8
8	467	172	64	20	8	755	276	93	28	10
7	735	275	86	26	8	1127	436	138	37	13
6	1174	434	125	36	11	1794	704	212	53	18
5	2089	753	210	51	15	2725	1077	307	68	23
4	4322	1657	425	95	29	4001	1707	559	151	55
3	7086	3517	1500	646	297	5860	2522	898	231	93
2	10317	6063	3336	1594	787	9628	5503	2663	1072	487
1	14389	9750	6105	3435	2085	12939	8222	4273	1648	745

	Stage I DOWN					Stage III DOWN				
# of Tumors	2	3	5	10	20	2	3	5	10	20
10	18	10	2	0	0	36	16	9	2	0
9	48	26	8	2	1	73	23	10	4	1
8	117	40	14	3	1	138	37	15	4	1
7	233	74	25	11	1	274	55	21	7	1
6	491	121	46	17	2	611	116	41	12	2
5	1045	252	77	26	9	1274	230	61	19	5
4	2233	522	157	44	14	2499	470	119	29	12
3	4506	1411	398	126	59	4383	1073	246	55	20
2	8347	3527	1063	278	108	7319	2605	839	267	128
1	13238	7321	2831	800	295	11412	5431	1840	544	215

When we compare the number of aberrantly regulated genes in early (stage I/II) vs. late stage (stage III/IV) tumors, we found no statistically significant increase in the number of differentially expressed genes in the late stage tumors as compared to the early stage tumors. In addition, the identities of differentially expressed genes in the early and late stage tumors were similar. However, we do see that most of the aberrantly regulated genes in the early stage tumors are also aberrantly regulated in the late stage tumors and that the late stage tumors do have additional alterations usually not present in the early stage tumors. It is clear, however, that the early stage tumors that we have been analyzing have a large number of genetic alterations and that they are much more similar to late stage tumors than to normal ovarian epithelium. The reduced expression of 20 of 30 down-regulated genes (60%) was confirmed in a panel of early and late stage tumors (15 each) by semi-quantitative PCR using GAPDH as a control.

We also constructed a number of subtraction suppression hybridization (SSH) cDNA libraries to identify both up- and down-regulated genes in the ovarian tumors relative to normal ovarian epithelium. We found that many of these genes were the same as genes identified as being aberrantly regulated using transcriptional profiling, but we did isolate a number of additional genes which were not present on the microarrays. We are currently preparing secondary microarrays containing the genes which were identified as being aberrantly regulated on the microarrays or identified from the SSH cDNA libraries. These will then be used to analyze a large number of primary ovarian tumors.

PROJECT #2: Characterization of the role of amplified oncogenes in the development of familial and sporadic ovarian cancer. Fergus Couch, P.I.

Background: The specific goal of this project is to identify genes that are over-expressed and amplified during the development of ovarian cancer, and to use these genes to test the hypotheses (1) that gene amplification contributes significantly to the development and progression of ovarian cancer, and (2) that familial and sporadic ovarian tumors have different progression pathways. To achieve this goal we proposed to perform expression analysis of a number of ovarian tumors using gene expression array profiling in collaboration with Millennium Predictive Medicine (MPMx). Over-expressed genes were to be assessed for amplification by Southern blot analysis of ovarian cancer cell lines and tumors. Amplified genes would then be positioned in clusters on chromosomal maps in an effort to define novel and known amplicons associated with ovarian cancer. Next, the most frequently amplified and over-expressed genes were to be characterized as candidate oncogenes using transformation assays in collaboration with the Biological Function Core. As part of the project we also proposed to identify genes that are amplified and/or over-expressed in familial ovarian tumors in an effort to discriminate between the progression pathways of familial and sporadic ovarian cancer. We proposed to identify these genes by comparing the gene expression profiles of sporadic and BRCA1 or BRCA2 mutant familial ovarian tumors.

Hypothesis/Objective: These studies are being undertaken in order to identify amplified and over-expressed genes in ovarian tumors that may function as novel oncogenes that contribute to development and progression of familial and sporadic ovarian cancer.

Relevance to Ovarian Cancer: These studies are designed to facilitate identification of novel amplified and over-expressed oncogenes in ovarian tumors. These genes likely make important contributions to ovarian tumor formation and by characterizing their function we may develop a better understanding of the processes involved in ovarian tumor development. In addition, by identifying and characterizing these genes we can potentially design novel prognostic markers, and immuno- and pharmacological therapies.

Methodology and Design:
Aim 1: To assemble collections of familial and sporadic ovarian tumors. The Mayo Clinic Ovarian Tumor Database will be screened in order to identify tumors from patients with a family history of breast and ovarian cancer. These tumors will then be screened for mutations in the BRCA1 and BRCA2 genes using a combination of CSGE and DHPLC mutation detection techniques. The goal is to identify 15-20 tumors that contain BRCA1 and BRCA2 mutations. As 10% of all ovarian tumors have mutations in the BRCA1 and BRCA2 genes, we will add some additional candidate tumors for mutation screening in order to identify sufficient mutation carriers. These additional tumors will be selected from the pool of high grade serous ovarian tumors because a high proportion of ovarian tumors with BRCA1 and BRCA2 mutations are known to fit into this histological category.

Aim 2: To assess the role of gene amplification in progression of familial and sporadic ovarian cancer. A total of 15 sporadic and 15 familial ovarian tumors will be screened for amplification of 55 known oncogenes using the AmpliOnc array system from Vysis Inc. In addition CGH analysis of the ovarian tumors will be performed in order to identify all the regions of chromosomal gain and amplification in the tumors.

Aim 3: To identify novel amplified oncogenes in familial and sporadic ovarian tumors. A combination of expression analysis of 30,000 gene arrays and subtraction suppression hybridization of the sporadic and familial tumors will be used to identify genes that are up-regulated or over-expressed in the familial and sporadic ovarian tumors in collaboration with Millennium Predictive Medicine and the investigators from Project 1. Array profiles will be compared with normal ovarian epithelial tissue profiles and the most commonly and most significantly up-regulated genes will be selected. By comparing the chromosomal position of these genes with the CGH results, the genes most likely to be amplified will be identified. These up-regulated genes will be tested for amplification by Southern blotting of a panel of 30 ovarian tumors and cell lines. Other genes in the same area as amplified genes will then be investigated as targets of amplification in order to define the structure of the amplicon and to identify all candidate oncogenes in each region.

Aim 4: To characterize the role of the candidate oncogenes in ovarian tumor development by functional analysis and to assess the clinical significance of these genes. Over-expressed and amplified genes identified in this manner will be assessed for a role in ovarian tumor development and progression. This will be assessed using transformation assays of ovarian cell lines and mouse xenograft models in association with the Biological Function Core. Full-length expression constructs will be generated and then transfected into normal ovarian epithelial cell lines. Stable transformants will be measured for increases in growth rate, colony formation, anchorage independence, and tumor formation in nude mice. Amplified and over-expressed genes that alter the growth rate of cell lines or tumors will be classified as oncogenes.

Results:

Task 1: We have identified a greater number of ovarian tumors with family history than previously expected. In total we have selected 50 of these tumors and an additional 25 high-grade serous ovarian tumors for mutation screening of the BRCA1 and BRCA2 genes. The delay in completing this task is due to additional efforts in review of clinical charts of patients in order to maximize the identification of tumors with associated family history of breast and ovarian cancer. Using these chart reviews we have identified 3 tumors with known BRCA1 and BRCA2 mutations. In addition, we have now completed PCR amplification of all 32 primer sets for the BRCA1 gene on each of the 75 tumors. The next stage is to analyze these amplification products for mutations using CSGE and DHPLC. We have recently acquired a DHPLC apparatus and will preferentially use this automated method for screening. Because of the large number of tumors that are associated with a history of breast and ovarian cancer and are likely to contain mutations, we no longer plan to carry out LOH studies.

Task 2: To date we have completed CGH analysis of 25 ovarian tumors. These are predominantly late-stage and high-grade serous ovarian tumors. CGH profiling of an additional 15-20 tumors is ongoing. No AmpliOnc array studies of the tumors has yet been carried out because we must first identify 15 tumors that carry BRCA1 and BRCA2 mutations. However, the system has been established in the Molecular Cytogenetics Core.

Task 3: To initiate this task we selected and processed a large series of ovarian tumors from the Mayo Clinic Tumor Bank. This involved H+E analysis and pathology review of 125 tumors by Dr. Roche in the Tissue Acquisition and Processing Core. A total of 60 of these tumors have been selected for our ongoing studies with MPMx. Tumors were selected for further analysis if each tissue specimen contained greater than 75% tumor material, thereby limiting contamination by other non-tumorigenic cells. These 60 tumors of all histological types, stages, and grades have been forwarded to MPMx where they will be profiled on gene expression arrays by MPMx staff and by Dr. Shridhar. To date Dr. Shridhar from Project 1 has generated 3 subtraction libraries and has completed 4 gene expression array profiles on the same ovarian tumors that have been used for CGH analysis. The array data from tumors and normal ovarian epithelial cells has been compared, and the genes that are most frequently over-expressed in the 4 tumors have been selected. In addition, genes identified as up-regulated in tumors by subtraction hybridization have been added to this list of genes. The addition of more gene array profiles will allow us to select the most frequently over-expressed genes that can then be cross-referenced with CGH profiles to identify genes for analysis of amplification.

Meanwhile, we have developed a strategy aimed at detecting regions of amplification in single tumors. We have used a moving window analysis of the gene expression array data to plot the median expression level of ordered sets of 10 genes along each chromosome. If a cluster of genes in a region is amplified, then several of these genes would be expected to be commonly over-expressed. Thus, by identifying peaks on the moving window plot, we can select candidate regions of amplification. To date we have detected amplification and over-expression of the Her2/neu and c-Myc oncogenes in 2 of the 4 profiled tumors. In addition, several novel regions of possible amplification have been selected that coincide with regions of chromosomal gain detected by CGH. These regions await validation of amplification by Southern blotting of ovarian tumor and cell line DNA using gene specific probes.

Conclusions:
The results obtained thus far suggest that this study will be successful in identifying a number of novel amplified genes in ovarian tumors. We have already shown that we can predict amplification of the Her2/neu and c-Myc genes using a combination of gene expression array profiling and CGH. By completing these analyses on a larger number of tumors, we will be able to select the most frequently amplified genes in ovarian tumors and begin to characterize their role in ovarian cancer. As a result we expect to develop a much improved understanding of the genes involved in development and progression of ovarian tumors.

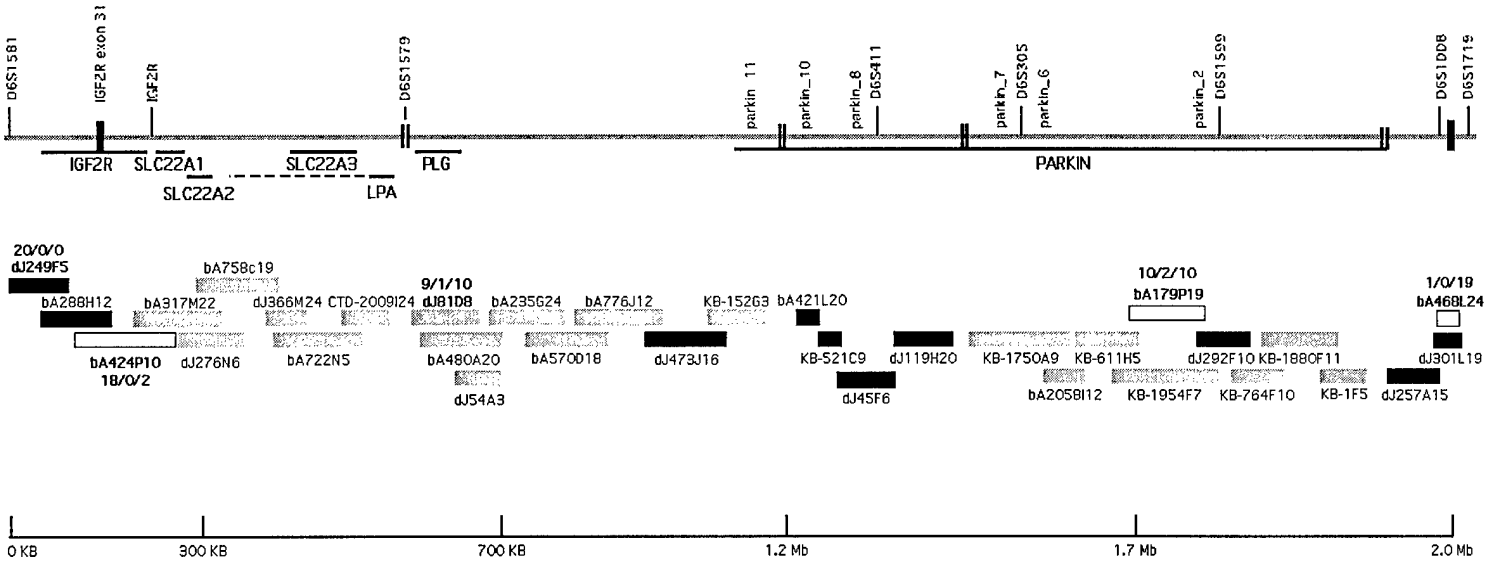
PROJECT #3: Common Fragile Sites and Ovarian Cancer. David I Smith, P.I.

There were four major specific aims to this proposal. **Specific Aim #1:** The cloning and characterization of FRA6E (6q21) and FRA6F (6q26) as these two common fragile sites are derived from chromosomal regions frequently deleted during the development of ovarian cancer. **Specific Aim #2:** The isolation of genes from the FRA6E and FRA6F regions, followed by examining each of them as potential tumor suppressor genes or as sensors of genomic damage. **Specific Aim #3:** The third specific aim was to perform clinical correlative studies in ovarian cancer with our impressive resource of fresh frozen ovarian tumors with full clinical follow-up. **Specific Aim #4:** The final specific aim, and the one that linked this project to the other projects with this Program Project Grant, was to characterize aberrantly expressed genes derived from chromosomal bands containing common fragile sites, to determine if these genes actually do reside within common fragile site regions. We feel that we have made excellent progress towards the completion of these goals. The most significant aid to all our work, however, has been the rapidly maturing human genome mapping and sequencing efforts, which has greatly facilitated

our ability to map and quickly characterize chromosomal regions and the genes contained within. This may have a dramatic effect on our ability to more rapidly complete our stated goals, especially since many of the steps that we proposed to do using classical molecular biological techniques can now be done electronically.

Specific Aim #1: Our first goal was to analyze two chromosomal regions on chromosome 6 that are frequently deleted in ovarian tumors and that contain two of the common fragile sites. Common fragile sites are specific chromosomal regions that form nonrandom gaps or breaks when cells are exposed to specific chemical conditions such as chemicals that inhibit DNA replication. These regions were 6q21 (FRA6F) and 6q26 (FRA6E). Since loss in these two regions occurs in up to 80% of advanced stage ovarian cancer, we were interested in analyzing the genes within the regions most frequently lost to see if they played an important role in ovarian cancer development. We have now more fully characterized the FRA6E common fragile site and find that it covers a large region, similar to several of the other characterized common fragile sites. We have a BAC contig surrounding this region and the BACs were then used as FISH-based probes to determine that this common fragile site spans a region of at least 800 Kb.

Specific Aim #2: We have (mostly electronically) mapped a number of relatively average sized genes within the FRA6E region. In contrast to the FRA3B and FRA16D fragile sites, which each contain a single large gene spanning the entire fragile site region, FRA6E contains at least 8 distinct genes. We know the complete genomic structure of each of these genes, and we have begun to analyze their expression in our ovarian cancer cell lines and primary ovarian tumors. Preliminary results reveal that several of these genes are frequently not expressed in ovarian tumors. The figure below shows a map of the FRA6E region and the genes identified to date within this region.



Specific Aim #3: The goal of this specific aim is to perform clinical correlative studies with the genes identified within the common fragile sites. Since we are still in the process of identifying these genes, we have done very little work towards completing this specific aim.

Specific Aim #4: The goal of this specific aim was to see if any of the consistently down-regulated genes identified in the transcriptional profiling work of Dr. Shridhar were derived from previously uncharacterized common fragile site regions. A number of the genes already localized to common fragile sites including FHIT (FRA3B), WWOX (FRA16D) and TESTIN (FRA7G) have been shown to

be frequently under- or not-expressed in tumors. We thus decided to see if searching for down-regulated genes derived from chromosomal regions containing common fragile sites might serve to quickly localize and characterize additional common fragile sites and the genes contained within them. We analyzed the data generated by Dr. Shridhar (Project #1) and found that many of the genes frequently not expressed in ovarian tumors are actually derived from the chromosomal regions that contain common fragile sites. We analyzed 10 genes and have found that 7 of them localize to previously uncharacterized common fragile sites. This work suggests that many of the genes contained within the common fragile sites are inactivated during the development of ovarian cancer. Our next step will be to determine what additional potentially important genes reside within the common fragile sites. The genes within the common fragile sites are currently being analyzed within the Biological Function Core to determine their role, if any, in the development of ovarian cancer. We are excited about this work as it demonstrates that the common fragile sites are somehow involved in the development of ovarian cancer and that there are potentially a number of very interesting genes residing within these sites whose inactivation is important for the development of this lethal disease. An important by-product of this effort is that we have now localized, and are in the process of characterizing, several additional common fragile sites.

ADMINISTRATIVE CORE

Background. The premise of our entire program project grant is that ovarian cancer develops upon a background of significant genetic alterations and that we can combine several powerful strategies, based in a rich tissue repository, to clone many of the genes involved in ovarian cancer development.

Objective. The overall purpose of the Administration Core is to support and oversee the work done by all three projects and the three other cores.

Relevance to Ovarian Cancer. There are ample data that frequent genetic alterations underlie the development of epithelial ovarian cancer. This is the premise underlying our entire program project grant. The Administration Core integrates all our efforts to (1) determine which of these myriad genetic abnormalities are relevant and (2) identify the biologic function of the relevant alterations.

Support Provided to Research Projects by Administration Core. The Administration Core performs these functions:

- Provide access to tissue repository
- Provide clinical and follow-up data for tissue specimens
- Link tissue and clinical data via a relational database
- Provide statistical support to all projects and cores
- Oversee all projects and cores including budgets
- Manage all human subject issues including approvals through the Mayo Institutional Review Board and the Department of Defense Human Subjects Reviews Groups; address all patient/family questions about research participation
- Organize meetings of investigators and external advisors
- Maintain a liaison with education and outreach specialists within the Mayo Women's Cancer Program and Mayo Clinic Cancer Center (MCCC)
- Formalize a partnership with representatives of the advocacy community

Methodology and Design. Our methods for the repository/database and statistical support are described here and in the following Results section.

Tissue repository. The Ovarian Research Group within the MCCC has stored specimens of a variety of ovarian tumors since 1991. The clinical information to match the tissue specimens is currently being abstracted from the medical records – this will include key risk factors such as family history and follow-up data. In the Results section below, we provide the current status of the tissue repository, clinical information, and relational database. Regarding statistical support, Dr. Steve Iturria in the Section of Biostatistics has provided statistical support for the genetic analyses. His duties have included maintaining all data files in a secure environment, performing diagnostics to ensure data quality, extracting data sets for analyses, and assisting investigators in the identification and use of appropriate statistical methods.

Results

Tissue repository. At the present time, we have over 800 epithelial ovarian tumors in a fresh frozen state.

Dr. Gary Keeney, gynecological pathologist in Laboratory Medicine and Pathology, has almost completed (91.5% reviewed) a formal review of all slides. His review has significantly decreased ambiguity about tumor morphologies and grades, and has allowed identification and exclusion of "ovarian tumors" deriving from a non-ovarian primary cancer.

Tumors that are to be used in assays are first sectioned and stained with hematoxylin and eosin. Dr. Pat Roche, Laboratory Medicine and Pathology and Head of the Tissue Acquisition and Processing Core Facility, looks at each slide and determines the percentage of the frozen section that is comprised of malignant cells. Only samples containing >70% tumor are used for analysis. Because of a consolidation of tumor acquisition and processing protocols within the last year, new tumors are screened prospectively for the percentage of the block comprised of malignant tissue. This will make decisions about use of specific tumor blocks more efficient in the future.

Chart abstraction for clinical follow-up. To date, we have abstracted 241 charts and this work is proceeding on schedule.

Relational database. At the present time, the details of our ovarian tissue collection are housed in an Access database, created to store information by tumor code numbers (patient identification is not distributed to individual investigators or cores). The clinical data and follow-up information are housed in a clinical database. At present, we are beginning the work to join the clinical database and the Access database in a web-based relational database. This project has been approved by the MCCC's Clinical Research Committee and is ready for programming to begin. Meetings are underway with statisticians and programmers to identify field characteristics and organizational structure for the data programmers and means of incorporating the necessary database security levels. The MCCC anticipates selection of an inventory/tracking system from three candidates by the end of 2000. This system will be incorporated into our relational database to expand capabilities for on-line sample handling documentation.

Statistical support. The statistical support that Dr. Iturria has provided thus far to the specific projects includes:

(Project #3) Examined the down-regulation of genes near known fragile sites in ovarian cancer. Tumor vs. normal expression ratios for genes lying in known fragile sites were contrasted with those of genes not lying in known fragile sites. Potentially unknown fragile sites were identified by constructing moving average plots for the tumor vs. normal expression ratios on each chromosome.

(Project #2) Identified over-expressed genes in ovarian cancer. Cluster analyses were performed to identify sets of genes that had common expression profiles across multiple experiments. Clusters, which exhibited a pattern consistent with over-expression in tumors, were identified. Various other gene-ranking methods were used to identify genes demonstrating significant increased expression in ovarian tumors. Moving average analyses were carried out to identify entire regions of gene amplification.

(Project #1) Identified under-expressed genes using methods similar to those used above.

Oversight. Drs. Smith and Hartmann communicate multiple times per week to ensure the fluid progress of all the projects and cores. They each interact independently with project and core leaders to address issues as they arise.

Human subjects issues; patient/family contact. We have developed a protocol for direct patient contact as we continue to build our tissue/clinical data repository. The steps for this protocol are as follows:

A. Tumor sent from Surgical Pathology to Tumor Acquisition Core Facility.
B. Tumor Acquisition Core sends e-mail notification of tumor received to Study Coordinator.
C. Surgical pathology results reviewed to determine appropriate participation level.
D. Patient contacted by Study Coordinator regarding participation in Ovarian Tumor Study.
E. Consent form signed, blood request ordered, and questionnaire completed.
F. Materials filed in master file along with Patient Enrollment Master Sheet.

All patient/family contact materials have been approved by the Mayo IRB and the Department of Defense Human Subjects Review Group (although it took considerably longer to obtain approval from the DOD to initiate this work).

Coordination of meetings. Our Ovarian Cancer Research group meets the third Monday of every month from 10:30 a.m.–12:00 noon. Fifteen to 20 individuals attend, including the project leaders, core leaders, technicians, and trainees involved in this ovarian cancer effort. At these meetings investigators present their scientific data for discussion, and new questions and collaborations are generated. Moreover, our Executive Committee meets monthly to assure smooth operations among the projects and within the MCCC. Dr. Hartmann meets weekly with the study abstracter and the laboratory personnel involved in sample acquisition.

Maintain liaison with education and outreach specialists within the Mayo Women's Cancer Program and MCCC. Ms. Julie Quam is the nurse coordinator for the Women's Cancer Program and routinely attends our scientific sessions as does Ms. Lisa Copeland, MCCC Communications. The issues of gynecologic cancer patients are routinely addressed at our MCCC public education events which occur in the fall and spring each year. Examples of recent brochures are available upon request.

Formalize a partnership with representatives of the advocacy community. In the past year, we have hosted visits for Ms. Patricia Goldman, Ms. Betty Reiser, and Ms. Deborah Collyar. Ms. Goldman and Ms. Reiser are prominent ovarian cancer advocates nationally. Ms. Collyar is the President of Patient Advocates in Research. Mayo investigators are working closely with the Minnesota Ovarian Cancer Alliance (MOCA) as it develops its organization. Eleven Mayo staff members serve on the MOCA Medical Advisory Board. We are committed to incorporating direction and guidance from the patient advocacy community as we conduct our research and map out strategies for future studies.

Conclusions
We have built upon our prior infrastructure in women's cancers within the MCCC to upgrade our database, mobilize needed resources to investigators in a timely manner, and provide a smooth organizational structure for the conduct of our ovarian cancer genetics research.

TISSUE ACQUISITION CORE FACILITY

The Tissue Acquisition Core provides a coordinated, centralized, and dedicated program for procurement and processing of biospecimens obtained from ovarian cancer patients and from populations of women at risk of developing ovarian cancer. Clinically annotated human biospecimens have historically been one of the most valuable and unique resources available for translational research at Mayo Clinic Rochester and the GOAL of the Core is to procure a tissue and/or a blood specimen from every ovarian cancer patient seen at Mayo Clinic. The Core will coordinate acquisition of both normal and neoplastic ovarian tissues, will process blood samples, will provide investigators with DNA and RNA, and will continue to bank biospecimens for future translational research. The Core will also serve as a resource of expertise, collaborative support, and service for pathology, immunohistochemistry, *in situ* hybridization, laser capture microdissection, RT-PCR, and digital image analysis. The Core will interface and be electronically integrated with the Ovarian Cancer Patient Registry and the Biostatistics Core to provide investigators clinically annotated biospecimens. The collection, banking, and use of biospecimens will be performed with appropriate patient consent and institutional approval. The Core will interact and collaborate with other federally-funded Ovarian Cancer Programs to promote resource sharing and integrate scientific projects.

MOLECULAR CYTOGENETICS CORE FACILITY

We are extremely fortunate to have an outstanding molecular cytogeneticist working with our ovarian Program. Dr. Robert Jenkins is responsible with starting with Ovarian Program (with Dr. Hartmann) and he has done much work on the characterization of many of the ovarian tumor specimens already collected. We did not request any funding from the Department of Defense to support this Core, as it is supported by the Mayo Clinic Cancer Center. However, we still expected the Core to provide support for routine cytogenetic and molecular cytogenetic services to members of the Ovarian Cancer Program. This Core has provided excellent service to the Program Project as we have been able to use comparative genomic hybridization to characterize 25 of the ovarian tumor specimens. We will also be transcriptionally profiling the same ovarian tumors, and thus will have both expression and cytogenetic information on the same set of tumors.

The purpose of the Biological Function Core is to provide normal ovarian epithelial specimens for the studies outlined in Projects 1-3 and assess the functional consequences of the genetic alterations detected during completion of Projects 1-3. To perform this analysis, we proposed to: (1) generate new cell lines from primary ovarian cancers and normal ovarian epithelium; (2) transfect these cell lines with appropriate plasmids to recapitulate the genetic alterations identified in Projects 1-3; (3) assess the effects of this transfection on proliferation rate, clonogenicity, and ability to form tumors in nude mice; (4) determine the effects of the transfected constructs on sensitivity of cell lines *in vitro* to agents commonly used to treat ovarian cancer; and (5) examine the effects of the genetic alterations on the therapeutic modalities when control or transfected cells are grown as xenografts in nude mice.

Hypothesis: The functional studies are being undertaken to determine whether the genetic alterations detected in ovarian cancer cells alter the proliferative rate, apoptotic threshold, and/or drug sensitivity of the tumor cells *in vitro* and *in vivo*.

Relevance to ovarian cancer: These activities are designed to: i) provide additional samples and models that can be used to study the biology of ovarian cancer vs. normal ovarian surface epithelium, ii) demonstrate how individual genetic alterations contribute to the cancer phenotype, and iii) potentially identify new gene products that can be investigated as possible therapeutic targets.

Core Facility Support Provided to Research Projects: As indicated below, the Biological Function Core has provided normal ovarian surface epithelial cells as normal controls in support of Projects 1-3. In addition, the Core has taken a gene identified in Project 3 and demonstrated its effect on drug sensitivity. Finally, the Core has begun to develop cell lines and animal models required for assessment of additional genes that are in the process of being identified in Projects 1-3.

Results: In the four months since funding was received for this program project grant, substantial progress has been made by the Biological Function Core.

Task 1: Provision of normal ovarian surface epithelial cells for study controls. Uncultured brushings of normal ovaries (documented to contain sheets of normal ovarian surface epithelial cells) and cultures of ovarian surface epithelium have been provided to Millennium Predictive Medicine as well as the Smith and Couch laboratories to provide normal controls for Projects 1-3. In addition, we have successfully introduced a construct encoding a temperature-sensitive SV40 large T antigen into normal ovarian surface epithelial cells. The resulting cells proliferate only at the permissive temperature and are highly transfectable, providing a new model system in which to explore the biological function of genes found to be altered in Projects 1-3.

Task 2: Optimization of current cell lines and development of new cell lines. As indicated below, many functional studies will involve expression of cDNA encoding a particular transcript into cells that have low levels or lack that transcript. To facilitate these studies, we optimized the transfection efficiency and geneticin-induced killing in each of six currently available low-passage ovarian cancer cell lines. We have also inserted a doxycycline sensitive transcriptional regulator into these six lines so that potentially toxic genes can be expressed in these cell lines in a conditional fashion. Finally, we attempted to develop additional ovarian cancer cell lines. To date we have attempted to culture 66 ovarian cancer specimens, 32 of which were primary epithelial tumors of serous or endometrioid morphologies. Although we did not observe spontaneous immortalization in any of these specimens, we have been able to freeze low passage cultures on 10 samples for potential immortalization in the future.

Task 3: Assess the effect of genetic alterations on proliferation rate and cloning efficiency in vitro. Work of Dr. Shridhar has resulted in the identification of MC-J, a DNA-J homolog whose 1.2 kb transcript is absent from 70% of ovarian cancer specimens. Further analysis has indicated that the down-regulation results from gene hypermethylation rather than mutation or deletion. To assess the functional consequences of MC-J down-regulation, the OV167 ovarian cancer cell line (which lacks MC-J expression) was transfected with MC-J under the control of the constitutive cytomegalovirus promoter or with empty vector. The growth rates of parental cells, empty vector controls, and two separate cloned transfectants (both showing readily apparent MC-J expression by RT-PCR analysis) were indistinguishable. Likewise, the clonogenicity of OV167 was unaffected by MC-J expression.

Task 4: Assess the effect of genetic alterations on sensitivity to various treatments in vitro. To date, the parental OV167 cells, empty vector controls, and the two separate cloned MC-J transfectants were examined for sensitivity to cisplatin, paclitaxel, and topotecan *in vitro*. Sensitivity to cisplatin was unaffected by MC-J expression. In contrast, overexpression of MC-J enhanced sensitivity of the cells to both paclitaxel and topotecan in colony forming assays. This enhanced sensitivity was also reflected in a more rapid induction of apoptosis after addition of drugs to the cells.

Task 5: Assess the effect of genetic alterations on tumorigenicity and drug sensitivity in vivo. To date five of the six low passage ovarian cancer cell lines have been injected into nu/nu mice; and we are waiting to see whether tumors will grow. Further experiments to assess the effect of genes identified in Projects 1-3 (e.g., MC-J) on tumorigenicity and *in vivo* drug sensitivity are planned.

Conclusions: First, we have developed a potentially useful model of SV40-immortalized ovarian surface epithelial cells that might be useful for assessing the effects of certain genetic changes observed in ovarian cancer cells. Secondly, we have demonstrated that diminished expression of the protein MC-J, which is observed in a substantial fraction of ovarian serous adenocarcinomas, results in diminished sensitivity to paclitaxel and topotecan.

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Key Research Accomplishments

- Completed the transcriptional profiling using DNA microarrays containing 25,000 genes against 20 primary ovarian tumors.
- Constructed SSH cDNA libraries from several of the same tumors that were transcriptionally profiled. Several thousand clones from each of these libraries have been sequenced.
- Confirmed that 60% of the genes which appear to be down-regulated are indeed down-regulated in a panel of low-stage and high-stage ovarian tumors.
- Identified a number of ovarian tumors from patients with a family history of ovarian cancer. We have already identified three tumors with known BRCA1 or BRCA2 mutations.
- Completed comparative genomic hybridization analysis of 25 ovarian tumors, including the 20 tumors that were transcriptionally profiled.
- Cloned and characterized the FRA6E common fragile site. We have also localized at least eight different genes within this 800 Kb fragile site region. Several of these genes show a loss of expression in primary ovarian tumors.
- Found that many of the consistently down-regulated genes in the ovarian tumors are derived from chromosomal bands containing a common fragile site. We have used these genes to clone seven new common fragile sites.
- Integration of the work of our Ovarian Program with the outstanding Ovarian Group working at M.D. Anderson (Drs. Gordon Mills and Robert Bast).

Reportable Outcomes

Since the funding for this Program Project did not begin until August 2000, we have not had sufficient time to publish papers based upon our work. However, we are in the process of writing several papers including a paper on the characterization of MCJ and a paper comparing the transcriptional profiling of early vs. later stage ovarian tumors. We have submitted a number of abstracts based upon this work and have given a number of presentations describing work supported by the Department of Defense.

Manuscripts Published

- Muthusamy T, **Kaufmann SH, Couch FJ**. BRCA1 facilitates stress-induced apoptosis in breast and ovarian cancer cell lines. *J. Biol. Chem.* 275:33487-33496, 2000.

Manuscripts in Preparation

- **Shridhar V**, Lee J, Pandita A, Avula R, Staub J, Iturria S, Calhoun E, Sen A, James CD, Kalli K, Keeney G, **Couch F, Hartmann LC**, Mills G, Bast R, Lillie J, **Smith DI**. Comprehensive analysis of genetic alterations in early and late stage ovarian tumors by cDNA-microarray expression profiling, and comparative genomic hybridization.
- **Shridhar V**, Bible KC, Staub J, Avula R, Lee YK, Kalli K, Huang H, **Hartmann LC, Kaufmann SH, Smith DI**. Loss of expression of a new member of the DNAJ protein family confers resistance to chemotherapeutic agents used in the treatment of ovarian cancer.

Abstracts

- **Shridhar V**, Staub J, Huang H, Callahan G, Bright RK, Yokomizo A, Wang L, Pass HI, **Hartmann L, Smith DI.** Loss of expression by deletion and hypermethylation of a new member of the DNAJ protein family on 13q14.1 in ovarian cancer. *Amer. J. Hum. Genet.* 65:A1817, 1999.
- **Shridhar V**, Callahan G, Staub J, Avula R, **Hartmann LC, Smith DI**. Identification of novel genes not expressed in primary ovarian tumors and cell lines. *Cancer Res.* 41:A1983, 2000.
- Callahan G, **Shridhar V**, Bale LK, Kalli KR, **Hartmann LC**, Conover C, **Smith DI**. Loss of PAPP-A expression in ovarian epithelial carcinoma. *Cancer Res.* 41:A2214, 2000.
- Muthusamy R, **Kaufmann SH, Couch FJ**. Fas ligand and caspase-8 mediate BRCA1 induced apoptosis in breast and ovarian cancer cell lines. *Cancer Res.* 41:A3539, 2000.
- Denison SR, **Shridhar V**, Ferber MJ, Becker NA, Callahan G, Lee J, Lillie J, **Smith DI**. Cloning and characterization of FRA6E. *Amer. J. Hum. Genet.* 67:A110, 2000.
- Ferber MJ, Denison SR, Becker NA, Lee J, Lillie J, **Hartmann LC, Shridhar V, Smith DI.** Genes within the common fragile sites are down-regulated in ovarian cancer. *Amer. J. Hum. Genet.* 67:A447, 2000.
- **Smith DI**, Sen A, Avula R, Staub J, Lee J, **Hartmann L**, Lillie J, **Shridhar V**. Identification of differentially expressed genes in early and late stage primary ovarian tumors by the construction of subtraction suppression hybridization cDNA libraries. *Amer. J. Hum. Genet.* 67:A505, 2000.

Presentations by David I Smith

- The Ovarian Cancer Research Program of the Mayo Clinic Cancer Center. Presented at Virginia Medical College, Richmond, VA. March 6, 2000.
- The Genetics of Ovarian Cancer in the Post-Genomics Era. Presented as the Distinguished Lecturer for the Women's Cancer Program at M.D. Anderson Cancer Center, Houston, TX. June 7, 2000.
- Transcriptional Profiling to Understand the Underlying Biology of the Development of Ovarian Cancer. Presented as the Distinguished Lecturer for the Women's Cancer Program at M.D. Anderson Cancer Center, Houston, TX. June 7, 2000.
- Transcriptional Profiling to Understand the Underlying Biology of the Development of Ovarian Cancer. Presented at Henry Ford Hospital, Detroit, MI. July 14, 2000.
- Common Fragile Sites and Cancer. Presented at the Common Fragile Sites, Gene Amplification and Cancer Meeting, Held at Mayo Foundation, Rochester, MN. August 25-26, 2000.
- Common Fragile Sites and Cancer. Presented at the Univ. of Nebraska in Omaha. Sept. 11, 2000.
- Common Fragile Sites and the Development of Cancer. Presented to graduate students at Mayo, Rochester, MN. September 27, 2000.

Conclusions

Although this project has only been funded for the past four months, we feel that we have made excellent progress towards the completion of our stated goals. Our collaborators at Millennium Predictive Medicine have provided us with access to microarrays containing 25,000 genes, and they have also sequenced thousands of cDNA clones derived from SSH cDNA libraries which Dr. Shridhar has constructed. We have used these arrays to characterize 20 primary ovarian tumors and we have constructed SSH cDNA libraries from several of these same tumors to identify additional genes not present on the cDNA microarrays. We are currently preparing secondary microarrays containing those genes which were found to be aberrantly regulated, either from the primary microarrays or from the SSH cDNA libraries. We have begun to identify tumors which contain BRCA1 or BRCA2 mutations and have begun to analyze these tumors (as well as all the tumors that were transcriptionally profiled) using comparative genomic hybridization analysis. We have cloned and characterized FRA6E and found that this fragile site is a large region (at least 800 Kb in size) that contains a minimum of eight genes. Several of these genes have been found to be consistently down-regulated in a panel of ovarian tumors. We have also found that several of the consistently down-regulated genes are derived from chromosomal regions containing common fragile sites and have been able to clone seven new common fragile sites by characterizing the regions surrounding these consistently down-regulated genes. The funding from the Department of Defense has been instrumental in bringing our Ovarian Program together and we are now a recognized clinical research program within the Mayo Clinic Cancer Center. We have begun to analyze the charts from all the patients who provided tumor samples and we are building our relational database. Finally our Biological Function Core has begun to analyze several of the aberrantly regulated genes identified. One very interesting gene, which is consistently down-regulated in ovarian tumors, has been shown to be involved in the development of resistance to chemotherapeutic agents routinely used in the treatment of this lethal disease.

What are the implications of this work? The major clinical problem with ovarian cancer is that most women present with late-stage disease and only 20% of these women will survive beyond five years. In contrast, women detected with early-stage disease have a much greater five-year survival. Our transcriptional profiling has identified a large number of consistently over-expressed genes in the primary ovarian tumors. A number of these are currently being tested by Millennium Predictive Medicine as suitable candidates for the early detection of ovarian cancer. A second major problem with ovarian cancer is that so little is known about the genetic alterations that underlie the development of this disease. The identification of consistently aberrantly regulated genes coupled with an analysis of the biological consequence of this misregulation (in our Biological Function Core) should help us to delineate important genes directly involved in the development of this poorly understood disease. We are also beginning to identify suitable numbers of ovarian tumors derived from patients with sporadic or familial ovarian cancer. The detailed comparison of expression profiles obtained from these two groups should enable us to determine if there are distinct pathways leading to sporadic as compared to hereditary ovarian cancer. We have also found that a number of the consistently down-regulated genes are derived from chromosomal bands containing common fragile sites. Thus, the common fragile sites and the genes contained within them may play an important role in ovarian cancer development.

We are not proposing any major changes at the present time from our original proposal. However, based upon our preliminary results in the characterization of the role of the common fragile sites in ovarian cancer development, we do propose to focus a much greater effort on the characterization of consistently down-regulated genes derived from common fragile site regions.

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BRCA1 Facilitates Stress-induced Apoptosis in Breast and Ovarian Cancer Cell Lines*

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The *BRCA1* tumor suppressor gene has previously been implicated in induction of high levels of apoptosis in osteocarcinoma cell lines. Overexpression of BRCA1 was shown to induce an apoptotic signaling pathway involving the c-Jun N-terminal kinase (JNK), but the signaling steps upstream and downstream of JNK were not delineated. To better understand the role of BRCA1 in apoptosis, we examined the effect of wild-type and C-terminal-truncated dominant negative BRCA1 on breast and ovarian cancer cell lines subjected to a number of different pro-apoptotic stimuli, including growth factor withdrawal, substratum detachment, ionizing radiation, and treatment with anticancer agents. All of these treatments were found to induce substantial levels of apoptosis in the presence of wild-type BRCA1, whereas dominant negative BRCA1 truncation mutants diminished the apoptotic response. Subsequent mapping of the apoptotic pathway induced by growth factor withdrawal demonstrated that BRCA1 enhanced signaling through a pathway that sequentially involved H-Ras, MEKK4, JNK, Fas ligand/Fas interactions, and caspase-9 activation. In addition, the pathway functioned independently of the p53 tumor suppressor. These data suggest that BRCA1 is an important modulator of the response to cellular stress and that loss of this apoptotic potential due to BRCA1 mutations may contribute to tumor development.

Mutations in the *BRCA1* tumor suppressor gene are found in many families with inherited breast and ovarian cancers and about half of families with a history of breast cancer only (1–3). *BRCA1* encodes an 1863-amino acid protein (1) that is located predominantly in the nucleus (4–6). This polypeptide has been implicated in the regulation of a wide variety of biological functions, including growth suppression, induction of apoptosis, cell cycle regulation, response to DNA damage, and maintenance of genome stability (7–11).

BRCA1 contains several well-defined functional domains. An N-terminal RING finger domain interacts with BARD1 (12), E2F transcription factor family members, cyclins and cyclin-dependent kinases (13). A domain in the middle of BRCA1 associates with the DNA repair protein RAD51 (10). The C-terminal BRCT domains are involved in transcription activation, growth inhibition and tumor suppression through interactions with RNA helicases, RNA polymerase II, TFIIH, TFIIE, BRCA2, and RAD51 (7, 14–18).

Several observations also support a role for BRCA1 in regulation of transcription. BRCA1 activates the $p21^{WAF1/Cip1}$ promoter in cells that contain wild-type or mutant p53 (19), suggesting that one of the mechanisms by which BRCA1 regulates cell cycle and suppresses growth is through the induction of p21. Additionally, BRCA1 binds the CtIP transcriptional repressor that inhibits BRCA1-mediated activation of the $p21^{WAF1/Cip1}$ promoter (20), interacts with CBP/p300 (21), and interacts with STAT1[1] to induce expression of the γ-interferon gene (*IFNγ*) (22). These data imply that BRCA1 might be directly involved in transcriptional activation of specific genes.

A growing body of evidence has also implicated BRCA1 in the preservation of genome integrity. Initial studies demonstrated that BRCA1 binds to the RAD50 and RAD51 DNA repair proteins (10, 23). More recent studies have implicated BRCA1 in transcription-coupled repair (24) and double strand DNA break repair (25). In accord with a possible role for BRCA1 in repair (or the control of repair), breast tumors from patients with *BRCA1* germ-line mutations contain 2- to 3-fold more chromosomal rearrangements than sporadic cancers.

Consistent with the proposed role of BRCA1 as a tumor suppressor, it has been observed that BRCA1 inhibits breast and ovarian cancer cell proliferation *in vitro* and in an experimental tumor model (7, 26). Conversely, selective reduction of BRCA1 mRNA levels using antisense RNA also induces more rapid cell growth and promotes cell transformation in NIH 3T3 fibroblasts (7). Whether the effects of BRCA1 on transcription and genome maintenance are sufficient to explain these results have been unclear. However, several recent reports have suggested that BRCA1 might also play a role in induction of apoptosis. Shao and colleagues (8) reported that expression of BRCA1 in mouse fibroblast and human breast cancer cell lines resulted in apoptosis in response to serum deprivation or calcium ionophore treatment. BRCA1-expressing prostate cancer DU-145 cells were subsequently shown to be susceptible to drug-induced apoptosis (27). More recently, Harkin and colleagues (28) demonstrated that BRCA1 expression can induce

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[1] The abbreviations used are: STAT1, signal transducers and activators of transcription 1; BSA, bovine serum albumin; dn, dominant negative; FACS, fluorescence-activated cell sorting; Fas, the cell surface receptor that is also designated Apo-1 or CD95; FasL, Fas ligand; GFP, green fluorescence protein; JNK, c-Jun N-terminal kinase, also known as stress-activated protein kinase (SAPK); MAPK, mitogen-activated protein kinase; PI, propidium iodide; z-VAD(OMe)-fmk, the methyl ester of *N*-(*N*$^{\alpha}$-benzyloxycarbonylvalinylalanyl)aspartate fluoromethylketone; MEK (MKK), mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEKK4, MEK kinase 4; MEF, mouse embryo fibroblast.

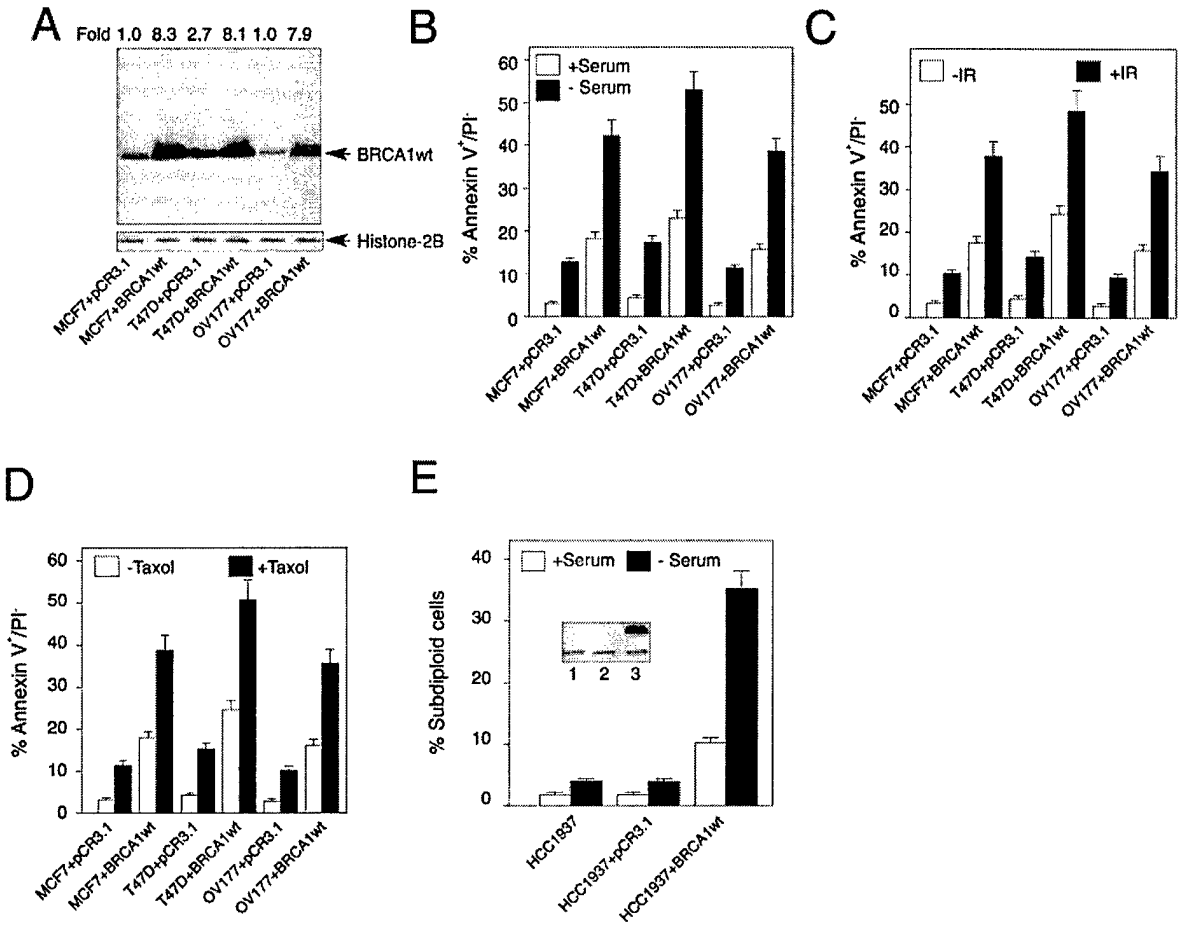

FIG. 1. **Transient expression of BRCA1 facilitates apoptosis in breast and ovarian cancer cell lines.** *A*, immunoblot analysis. Full-length BRCA1 cDNA expression construct or the pCR3.1 vector were transiently expressed in MCF7, T47D, and OV177 cells. After 24 h cell lysates were prepared. Aliquots containing 100 μg of protein were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with the C-terminal anti-BRCA1 (66046E) antibody. The -fold increase in BRCA1 expression in each cell line is shown above the blot. *B–D*, BRCA1 facilitates apoptosis in MCF7, T47D, and OV177 cell lines. Cells were transiently transfected with a BRCA1 expression construct or the pCR3.1 vector along with a GFP expression plasmid, incubated for 24 h, sorted by FACS for GFP expression, exposed to normal or serum-free medium for 24 h (*B*), ionizing radiation (*IR*) followed by incubation for 24 h (*C*), or paclitaxel for 24 h followed by incubation in drug-free medium for 24 h (*D*). At the conclusion of the incubation, cells were stained with Annexin V and PI. The proportion of Annexin V-positive/PI-negative cells was quantified by flow cytometry. *E*, BRCA1 facilitates apoptosis in BRCA1 null cell lines. HCC1937 BRCA1 null cells were transiently transfected with the BRCA1 or the pCR3.1 vector along with a GFP expression plasmid. The GFP-expressing cells were collected and subjected to immunoblotting (*inset*). *Lane 1*, represents untransfected cells; *lane 2*, pCR3.1-transfected cells; and *lane 3*, BRCA1wt-transfected cells. Sorted cells were also incubated for 24 h in the absence or presence of serum. After incubation, cells were fixed and labeled with PI. Quantitative analysis of subdiploid cells was performed by flow cytometry.

apoptosis through activation of a c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). In the present study, we have further delineated the relationship between BRCA1 and JNK-dependent apoptotic signaling in breast and ovarian cancer cell lines. We provide evidence that BRCA1 modulates stress-induced apoptotic signaling through a pathway that sequentially involves the H-Ras proto-oncogene, MEKK4, JNK, Fas (CD95)/FasL interactions, and activation of procaspase-8. In addition, we present evidence for dominant negative activity of BRCA1 mutants in the context of this apoptotic response.

EXPERIMENTAL PROCEDURES

Materials—Immunological reagents were purchased from the following suppliers: BRCA1 rabbit polyclonal antibodies directed to the C and N termini of BRCA1 (66056E and 66036E) from PharMingen; antibodies against caspase-8, caspase-9, Fas, Fas-L, phospho-JNK, and phospho-MEKK 1–4 from Santa Cruz Biotechnology; inhibitory anti-Fas antibodies ZB4 and Nok2 from Kamiya and PharMingen, respectively; p53 antibody from Oncogene Research Products; and alkaline phosphatase-conjugated secondary antibody from the Jackson ImmunoResearch Laboratories; z-VAD(OMe)-fmk was obtained from Enzyme Systems Products (Dublin, CA). An Annexin V apoptosis detection kit was purchased from R & D Systems (Minneapolis, MN). Propidium iodide (PI) and paclitaxel were procured from Sigma. An enhanced chemiluminescence kit was purchased from Roche Molecular Biochemicals.

Plasmids—The full-length BRCA1 coding sequence was subcloned into the pCR3.1 (Invitrogen) mammalian expression vector. Two truncation mutants of BRCA1 (BRCA1Δ5382-insC and BRCA1Δ5677-insA) were generated by polymerase chain reaction as described previously (19, 26). The GFP-encoding plasmid pEGFP-N-1 was from CLONTECH. MC159 and CrmA expression constructs were kindly provided by J. Bertin (National Institutes of Health) and C. Young (Mayo Clinic), respectively. Dominant negative expression plasmids dnMEKK1-HA-pCEPT4, dnMKK4-pRSET(Δ55–72), and dnMKK7-FLAG-pcDNA3 were provided by David McKean (Mayo Clinic). The dnJNK(APF)-pcDNA3 was kindly provided by Roger Davis. The Ras-N17 mutant construct was provided by Larry Karnitz (Mayo Clinic).

Cell Culture and Transfection—The human breast adenocarcinoma cell lines MCF7 and T47D were obtained from ATCC (Manassas, VA). The ovarian cancer cell line OV177 developed at the Mayo Clinic (29) was obtained from Cheryl Conover. The MCF7, T47D, and OV177 cells were grown in Dulbecco's modified essential medium, RPMI 1640, and α-minimal essential medium, respectively. Each of these was supplemented with 10% bovine calf serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine. Mouse embryo fibroblast (MEF) $p53^{-/-}$ and MCF7 HPV16 cells were grown in Dulbecco's minimal essential medium with 10% fetal bovine serum and the additives listed above. Cells were plated (1×10^6 cells) into 100-mm dishes,

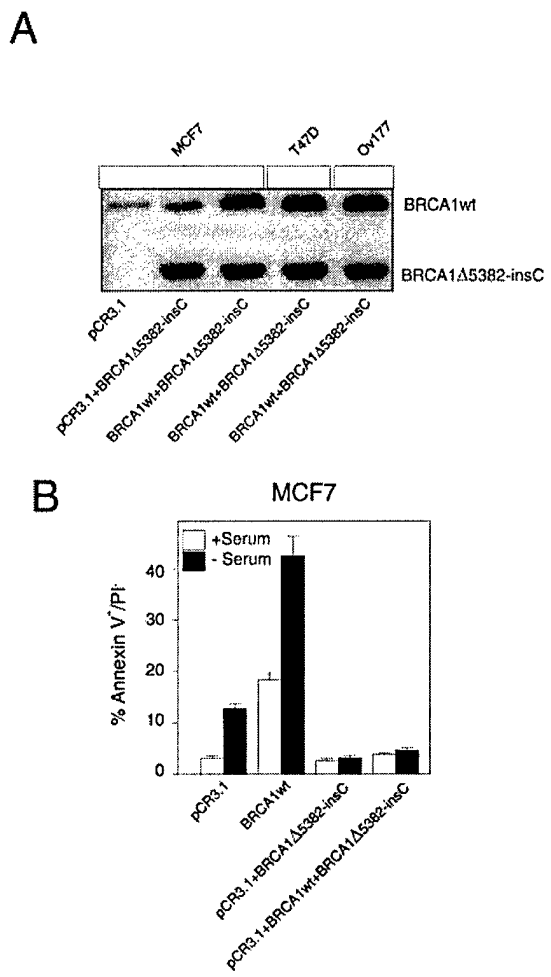

FIG. 2. **Truncated BRCA1 exerts an anti-apoptotic effect.** *A*, expression of the BRCA1Δ5382-insC construct results in formation of a stable truncated BRCA1 protein. BRCA1 wild-type (*wt*) and the BRCA1Δ5382insC mutant were transiently expressed in MCF7, T47D, and OV177 cells. Lysates were harvested 24 h after transfection. Aliquots containing 100 μg of protein from each cell line were probed using N-terminal BRCA1 (66036E) antibody. The wild-type BRCA1 protein is approximately 220 kDa and the C-terminal-truncated mutant BRCA1Δ5382-insC is 180 kDa in size. *B*, the BRCA1Δ5382-insC mutant inhibits BRCA1-dependent apoptosis. After transient co-transfection of full-length BRCA1 or the C-terminal-truncated BRCA1Δ5382-insC with a GFP plasmid, GFP-expressing MCF7 cells were incubated for 24 h in the presence and absence of serum, fixed, stained with PI and Annexin V and analyzed by flow cytometry.

allowed to adhere overnight, and then transiently transfected with 10 μg of either the full-length BRCA1 or mutant BRCA1 expression constructs along with 2.5 μg of the GFP expression plasmid, pEGFP-N1 using the Fugene 6 (Roche Molecular Biochemicals) transfection system. After 24 h, GFP-expressing cells were isolated by FACS and replated.

Induction of Apoptosis—Cells sorted for GFP expression were replated in 6-well plates and incubated in the presence or absence of serum for 24 h. Alternatively, the transfected cells were grown in 100-mm plates and subjected to 10 or 20 grays of γ-irradiation using a ^{137}Cs source (J. L. Shepard) delivering 103 centigrays/min. Cells were harvested after 8, 16, or 24 h. In some experiments, transfected cells were treated for 24 h with 100 nM paclitaxel, a concentration previously demonstrated to induce apoptosis in breast cancer cells (30).

Detection of Apoptosis—Cell death was determined by two-color flow cytometry after staining of cells with fluorescein isothiocyanate-labeled Annexin V and PI using a Becton Dickinson FacsCaliber. Cells that were Annexin V-positive and PI-negative were considered apoptotic. Apoptosis was also measured by quantitation of nuclei containing subdiploid amounts of DNA. In this latter method, cells were lysed in HFS (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml PI); subdiploid nuclei were counted by flow cytometry. For both of these flow cytometry methods, at least 10,000 gated events were recorded for each sample. Data were analyzed by Winlist and WinMDI software (Variety Software House, Topsham, ME).

Analysis of BRCA1 Expression—After transfection with empty vector, full-length BRCA1, or mutant BRCA1, cells were collected and lysed using EBC buffer (50 mM Tris-HCl, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate with protease inhibitors phenylmethylsulfonyl fluoride (100 μg/ml), aprotinin (20 μg/ml), and leupeptin (10 μg/ml)). For immunoblotting, equal concentrations (100 μg) of whole cell extract were loaded per lane, resolved in a 6% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. After blocking with 5% BSA in TBS-T (20 mM Tris-HCl, pH 8.0, 0.9% NaCl, and 0.05% Tween 20), membranes were incubated overnight at 4 °C with primary antibodies (66046E BRCA1 C-terminal and 66036E BRCA1 N-terminal) at a concentration of 2 μg/ml in TBS-T containing 1% BSA and 0.1% azide. After several washes with TBS-T, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG at 1:2000 dilution in 1% BSA/TBS-T. Signals were detected using enhanced chemiluminescence.

Cell Fractionation and Western Blotting—To assess the effect of BRCA1 overexpression on Fas, FasL, and caspases, aliquots containing 1×10^6 cells transfected with vector, full-length BRCA1, or BRCA1Δ5382-insC mutant, were incubated for 24 h in the presence and absence of serum. Cells were collected, washed in phosphate-buffered saline, and resuspended in cell lysis buffer containing 140 mM NaCl, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.2% Nonidet P-40, and the protease inhibitors aprotinin (80 ng/ml), leupeptin (40 ng/ml), pepstatin (40 ng/ml), chymostatin (40 ng/ml), and antipain (40 ng/ml). After incubation for 30 min on ice, the cells were homogenized and centrifuged at 14,000 × *g* for 15 min at 4 °C. The cell supernatants were resolved on SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose membranes, and incubated with primary and secondary antibodies.

RESULTS

Ectopic Expression of BRCA1 Facilitates Apoptosis in Breast and Ovarian Cancer Cell Lines—To study the functional properties of BRCA1, we transiently overexpressed full-length BRCA1 cDNA or pCR3.1 empty vector in two breast (MCF7 and T47D) and one ovarian (OV177) cancer cell line. Western blot analysis using the C-terminal anti-BRCA1 (66046E) antibody showed a 7- to 8-fold increase in BRCA1 protein on BRCA1 transfection when compared with the endogenous BRCA1 level in all three cell lines used in this study (Fig. 1*A*). To evaluate the effect of BRCA1 overexpression on cellular apoptosis, we cotransfected cells with BRCA1 or pCR3.1 and GFP, enriched for transfected cells by sorting for GFP-positive cells, incubated the transfected cells under various conditions for an additional 24 h, and then measured apoptosis using Annexin V and PI. Cells exposed to normal growth conditions (Fig. 1*B*, *+Serum*) demonstrated a 6- to 7-fold increase in Annexin V positivity when transfected with BRCA1 as compared with empty vector. For example, 17 ± 2% of BRCA1-transfected MCF7 cells were apoptotic, whereas only 2.5 ± 1% of vector-transfected MCF7 cells were apoptotic under the same conditions. These results suggest that increased levels of wild-type BRCA1 increase the rate of spontaneous apoptosis in the three cell lines.

To further examine the effect of BRCA1 expression on cellular apoptosis, cells were exposed to various apoptotic stimuli, including serum withdrawal, γ-irradiation, and treatment with paclitaxel. Staining with Annexin V and PI revealed that 13 ± 1%, 17 ± 2%, and 11 ± 1% of the vector-transfected MCF7, T47D, and OV177 cells, respectively, were apoptotic 24 h after removal of serum. In contrast, 42 ± 4%, 53 ± 4%, and 39 ± 3% of the BRCA1-transfected MCF7, T47D, and OV177 cells were apoptotic after serum withdrawal (Fig. 1*B*). Similarly, forced overexpression of BRCA1 enhanced the amount of apoptosis observed after treatment with γ-irradiation (Fig. 1*C*) and paclitaxel (Fig. 1*D*). These results suggest that expression of BRCA1 facilitates the response of breast and ovarian cancer cells to a variety of apoptotic stimuli.

Wild-type BRCA1 Is Required for Apoptosis—To further eval-

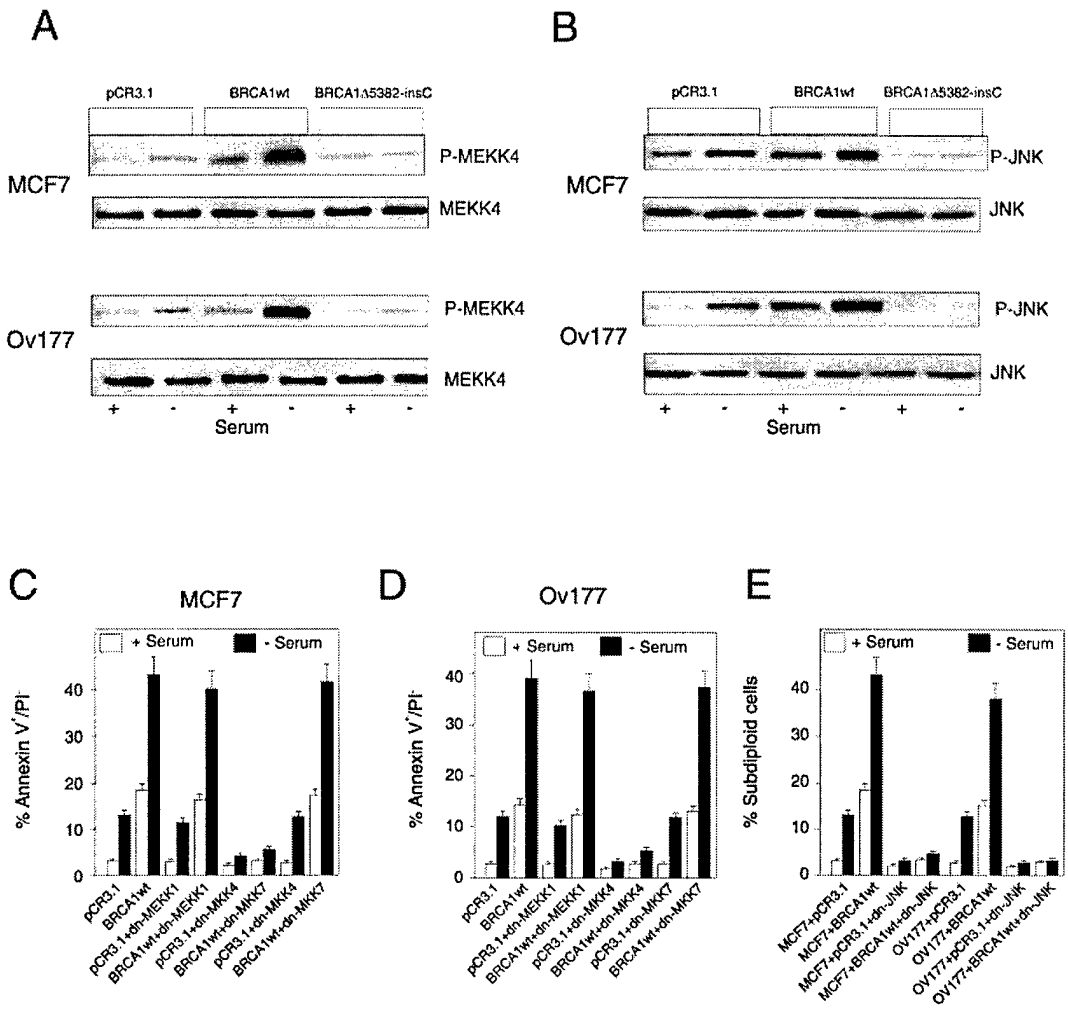

FIG. 3. **BRCA1-dependent apoptosis in response to serum withdrawal is mediated by a MEKK4, MKK4, and JNK signaling pathway.** *A* and *B*, phosphorylation of MEKK4 (*A*) and JNK (*B*) in MCF7 and OV177 cells transfected with pCR3.1 vector, full-length BRCA1, or BRCA1Δ5382-insC. Immunoblotting of 30 μg of protein from each transfection was performed with anti-phospho-MEKK4 or anti-phospho-JNK antiserum (*top panels*). To confirm equal loading, the membranes were reprobed with anti-MEKK4 or anti-JNK antibody (*bottom panels*). *C* and *D*, overexpression of dn-MKK4 but not dn-MEKK1 or dn-MKK7 inhibits BRCA1-dependent apoptosis. MCF7 cells (*C*) or OV177 cells (*D*) were co-transfected with dn-MEKK1, dn-MKK4, or dn-MKK7 and pCR3.1 or wild-type BRCA1 along with GFP. GFP-positive cells were purified by FACS and incubated in the presence or absence of serum for 24 h. At the end of the incubation, cells were labeled with annexin V and PI. *E*, overexpression of dn-JNK abolishes BRCA1-dependent apoptosis. MCF7 and OV177 cells were co-transfected with dn-JNK, GFP, and pCR3.1 or wild-type BRCA1. GFP-positive cells were purified and treated as in panels *C* and *D*.

uate the role of BRCA1 in apoptosis, we transiently transfected the wild-type BRCA1 and the pCR3.1 vector into HCC1937 cells, which express only the 5382insC-truncated form of BRCA1. When these cells were incubated in the presence and absence of serum for 24 h, the pCR3.1-transfected cells displayed only minor levels of apoptosis (<3%) (Fig. 1*E*). In contrast, ectopic expression of BRCA1 at levels that exceeded the expression level of the endogenous BRCA1Δ5382-insC-truncated protein (*inset*, Fig. 1*E*) increased the rate of apoptosis in the presence of serum to 10%, and to 38% upon serum withdrawal (Fig. 1*E*). The low levels of spontaneous and serum withdrawal-induced apoptosis in the absence of full-length BRCA1 suggest that BRCA1 plays an important role in this apoptotic process.

BRCA1 Mutants Abolish BRCA1-dependent Apoptosis—To further explore the role of BRCA1 in cellular apoptosis, we transiently transfected the BRCA1Δ5382-insC mutant construct, containing a common BRCA1 mutation found in BRCA1-associated breast and ovarian cancer families, into both breast and ovarian cancer cells. Immunoblotting with anti-BRCA1 66036E antibody, directed to the N-terminal portion of BRCA1, revealed that the truncated BRCA1Δ5382-insC protein was expressed at levels that equaled or exceeded the levels of ectopically expressed or endogenous wild-type BRCA1 protein (Fig. 2*A*). Under these conditions, the BRCA1Δ5382-insC protein diminished serum withdrawal-induced apoptosis associated with endogenous levels of BRCA1 (Fig. 2*B*). In addition, when expressed in stoichiometric amounts, the BRCA1Δ5382-insC mutant blocked spontaneous and growth factor withdrawal-induced apoptosis associated with ectopic expression of wild-type BRCA1 (Fig. 2*B*). Similar results were also observed in T47D and OV177 cells (data not shown). Furthermore, overexpression of another BRCA1 mutant (BRCA1Δ5677-insA) also abolished BRCA1-induced apoptosis in both breast and ovarian cancer cells (data not shown). These results clearly demonstrate that BRCA1 expression induces apoptosis in breast and ovarian cancer cells and that mutations in BRCA1 that commonly remove the C terminus, block this induction.

BRCA1 Facilitates Serum Withdrawal-induced Apoptosis by Enhancing Signaling through a Ras, MEKK4, and JNK Pathway—Harkin *et al.* (28) recently demonstrated that BRCA1-induced apoptosis is associated with activation of JNK/SAPK. However, signaling pathways upstream of JNK/SAPK, or the

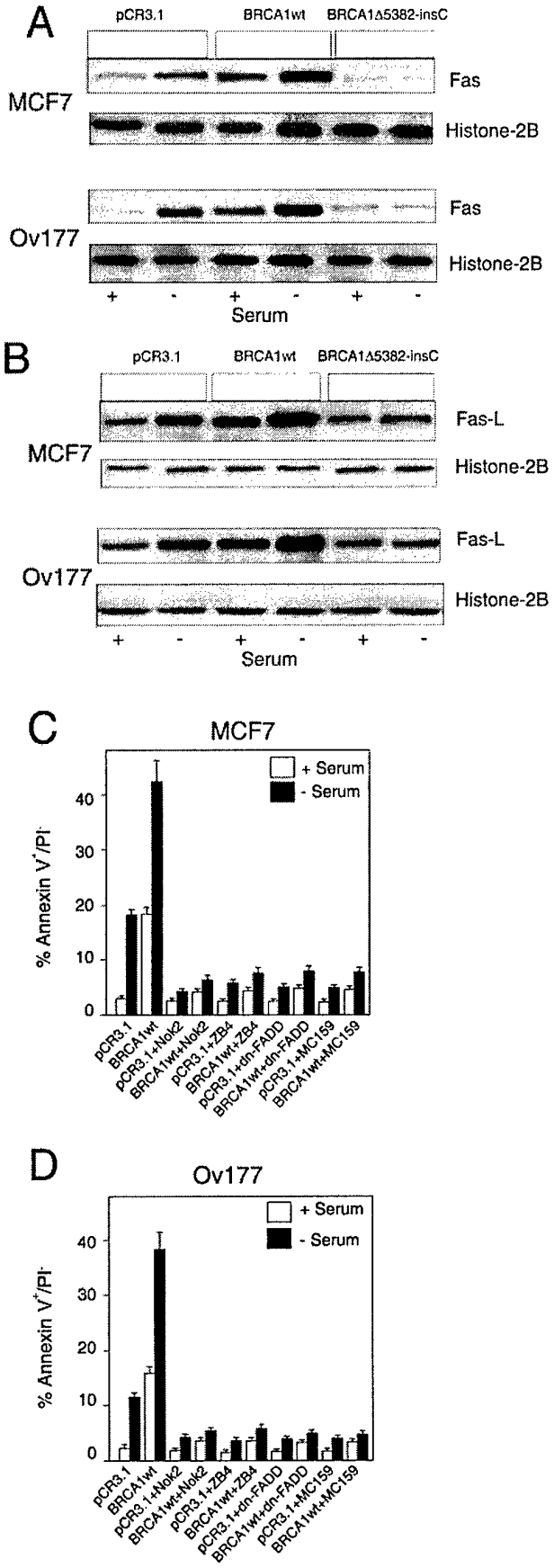

FIG. 4. **Fas and FasL mediate BRCA1-dependent apoptosis in breast and ovarian cancer cells after serum withdrawal.** *A* and *B*, analysis of Fas (*A*) and FasL (*B*) protein levels in MCF7 and OV177 cells transfected with GFP and pCR3.1 vector, wild-type BRCA1, or BRCA1Δ5382-insC. After GFP-expressing cells were incubated in the presence and absence of serum for 24 h, 30 μg of cell lysate was immunoblotted with anti-Fas antibody (*A*) or anti-FasL antibody (*B*). To confirm equivalent loading, blots were probed with anti-Histone H2B. *C* and *D*, inhibition of Fas signaling abolishes BRCA1-dependent serum withdrawal-induced apoptosis. MCF7 cells (*C*) or OV177 cells (*D*) were transiently transfected with GFP and BRCA1 or pCR3.1 in the absence or presence of plasmid encoding dnFADD or MC159. GFP-expressing cells were incubated for 24 h with Nok2 and ZB4 in the absence or presence of serum. Annexin V-positive/PI-negative cells were quantitated by flow cytometry.

downstream targets of JNK/SAPK, were not identified. To assess the role of JNK signaling in BRCA1-dependent serum starvation-induced apoptosis, we investigated the involvement of other components of MAPK signaling pathways. MCF7 and OV177 cells were transfected with pCR3.1 vector, full-length BRCA1 or BRCA1Δ5382-insC, sorted, and cultured in the presence and absence of serum for 24 h. Cell lysates prepared from these cells were subjected to immunoblotting with antisera that recognize phosphorylated species of MEKK1, MEKK4, and JNK. BRCA1 overexpression did not alter MEKK1 phosphorylation (data not shown) but did result in increased levels of phospho-MEKK4 and phospho-JNK, which were increased even further after growth factor withdrawal (Fig. 3, *A* and *B*). Conversely, the BRCA1Δ5382-insC mutant, which failed to induce apoptosis (Fig. 2*B*), failed to induce phosphorylation of these proteins (Fig. 3, *A* and *B*). Further evidence that activation of these kinases plays a critical role in BRCA1-dependent serum withdrawal-triggered apoptosis came from the observation that expression of dn-MKK4 or dn-JNK completely blocked BRCA1-dependent apoptosis in both MCF7 and OV177 cells in the presence and absence of serum (Figs. 3, *C–E*). In contrast, dominant negative forms of MEKK1 and MKK7 did not affect BRCA1-dependent apoptosis either in the presence or absence of serum (Fig. 3, *C* and *D*). These results suggest that JNK and its upstream activators MKK4 and MEKK4 are required for BRCA1-dependent apoptosis after serum withdrawal.

A number of activators of MEKK proteins have been identified previously, including Ha-Ras (31). Although Ras appears to play a major role in cell survival and proliferation, a role in induction of apoptosis has also been reported (32, 33). To investigate the role of Ras in BRCA1-dependent growth factor withdrawal-induced apoptosis, MCF7 and OV177 cells were transiently co-transfected with BRCA1 and a dominant negative form of H-Ras (Ras N17). Expression of Ras N17 resulted in complete abrogation of BRCA1-dependent apoptosis in the presence and absence of serum starvation (data not shown). Collectively, these observations suggest that BRCA1 facilitates apoptotic signaling through a Ras/MEKK4/MKK4/JNK pathway.

Fas (CD95/APO-1) Mediates BRCA1-dependent Serum Withdrawal-induced Apoptosis—Recent reports have suggested a role for Fas and FasL in neuronal apoptosis following nerve growth factor withdrawal (34). In addition, JNK has been shown to up-regulate the FasL promoter by activation of the c-Jun and ATF2 transcription factors (35). When combined with the results from the experiments described above, these observations suggest that Fas and FasL play a role in BRCA1-dependent apoptosis in response to growth factor withdrawal. To evaluate this hypothesis, MCF7 and OV177 cells were transiently transfected with pCR3.1, full-length BRCA1, or BRCA1Δ5382-insC, sorted, incubated in the presence and absence of serum for 24 h, and immunoblotted for Fas and FasL. BRCA1 expression up-regulated both Fas and FasL in MCF7 and OV177 cells (Fig. 4, *A* and *B*), and the levels of these proteins were further enhanced by serum withdrawal (Fig. 4, *A* and *B*). Conversely, BRCA1Δ5382-insC completely blocked induction of Fas and FasL by endogenous and ectopically ex-

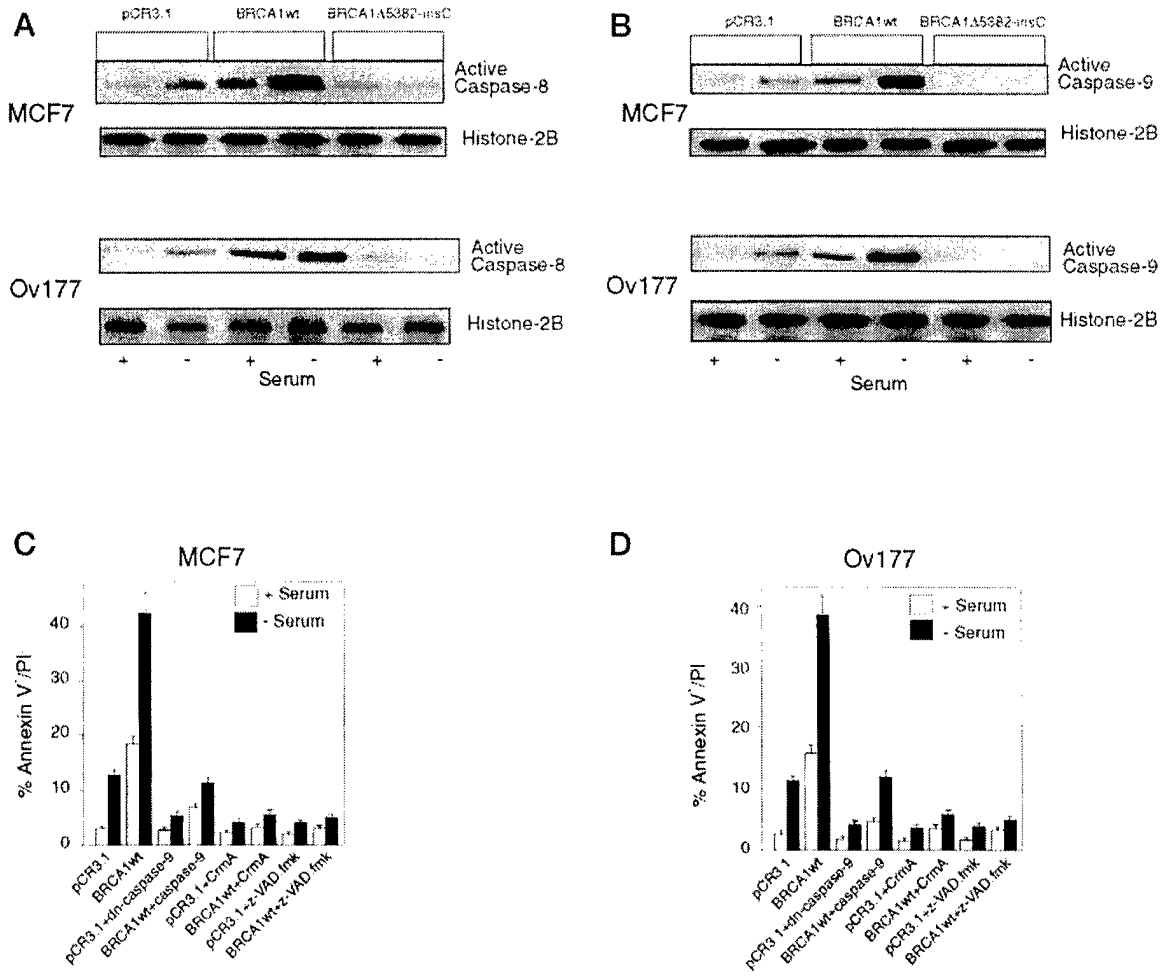

FIG. 5. **BRCA1-dependent apoptosis in serum-deprived breast and ovarian cancer cells is mediated by caspase-8 and caspase-9.** *A* and *B*, activation of caspase-8 (*A*) and caspase-9 (*B*) during BRCA1-dependent apoptosis in response to serum withdrawal in MCF7 and OV177 cells. Lysates from GFP-expressing cells transfected with GFP and pCR3.1, wild-type BRCA1, or BRCA1Δ5382-insC were probed with antiserum against active caspase-8 (*A*) or active caspase-9 (*B*). Blots were reprobed with anti-Histone 2B as a loading control. *C* and *D*, inhibition of caspase-8 or caspase-9 activity blocks BRCA1-dependent apoptosis in serum-deprived breast and ovarian cancer cells. MCF7 cells (*C*) or OV177 cells (*D*) were co-transfected with plasmids encoding GFP and dn-caspase-9 or CrmA along with pCR3.1 vector or full-length BRCA1. After GFP-expressing cells were isolated and incubated in the presence and absence of serum for 24 h, apoptosis was assessed by staining with Annexin V and PI. Alternatively, transfected cells were treated with z-VAD.fmk during the course of serum deprivation.

pressed BRCA1 in both cell lines (Fig. 4, *A* and *B*).

To further evaluate the role of Fas, Fas-L, and the adaptor protein FADD in this apoptotic signaling pathway, MCF7 and OV177 cells transfected with BRCA1 and the vector control were incubated with ZB4 or Nok2 in the presence and absence of serum. The Nok2 and ZB4 monoclonal antibodies bind to the Fas receptor and prevent cross-linking by Fas-L. Both of these antibodies abolished BRCA1-induced apoptosis in the presence and absence of serum (Fig. 4*C*). Likewise, co-expression of a dominant negative version of the adaptor protein FADD, or MC159, a *Molluscum contagiosum* viral inhibitor of FADD signaling (36), with BRCA1 abolished BRCA1-induced apoptosis (Fig. 4*C*). These results suggest that the BRCA1-dependent apoptosis in response to serum deprivation is Fas-dependent and that mutant BRCA1 blocks this apoptotic signaling pathway in both breast and ovarian cancer cells.

BRCA1-induced Apoptosis Is Caspase-dependent—Two pathways of Fas-dependent caspase activation have been described (37, 38). One involves recruitment of caspase-8 followed by direct activation of effector caspases, whereas the second involves activation of caspase-8 and cleavage of the Bcl-2 family member Bid to yield a fragment that induces release of cytochrome *c* from mitochondria and subsequent activation of effector caspases through the Apaf-1/caspase-9 pathway (39). To assess the activation of caspase-8 and caspase-9 in response to BRCA1 overexpression, lysates from MCF7 and OV177 cells transfected with pCR3.1, wild-type BRCA1, or BRCA1Δ5382-insC were immunoblotted with antisera directed to the cleaved, active forms of caspase-8 and caspase-9. BRCA1 overexpression resulted in cleavage of both caspase-8 and caspase-9 (Fig. 5, *A* and *B*). Serum starvation for 24 h resulted in further activation of both caspase-8 and caspase-9 (Fig. 5, *A* and *B*). As expected, the activation of caspase-8 and caspase-9 was completely blocked by co-expression of BRCA1Δ5382-insC (Fig. 5, *A* and *B*). In addition, the z-VAD.fmk general caspase inhibitor inhibited all BRCA1-dependent apoptosis. These results suggest that caspase-8 and caspase-9 are both activated during BRCA1-dependent apoptosis.

To further assess the role of these initiator caspases, we co-expressed CrmA, which selectively inhibits caspase-1 and caspase-8 (40), and a dominant negative caspase-9 construct, which inhibits caspase-9 activation (41) with BRCA1 and pCR3.1 vector in MCF7 and OV177 cells. Both CrmA and dn-caspase-9 abrogated BRCA1-induced apoptosis (Fig. 5, *C* and *D*), suggesting that the BRCA1-dependent signal is transduced through a caspase-9/Bid/Apaf-1/cytochrome *c*/caspase-9 (type II) pathway in both cell lines.

The MAPK Pathway Is Upstream of Fas Activation in the

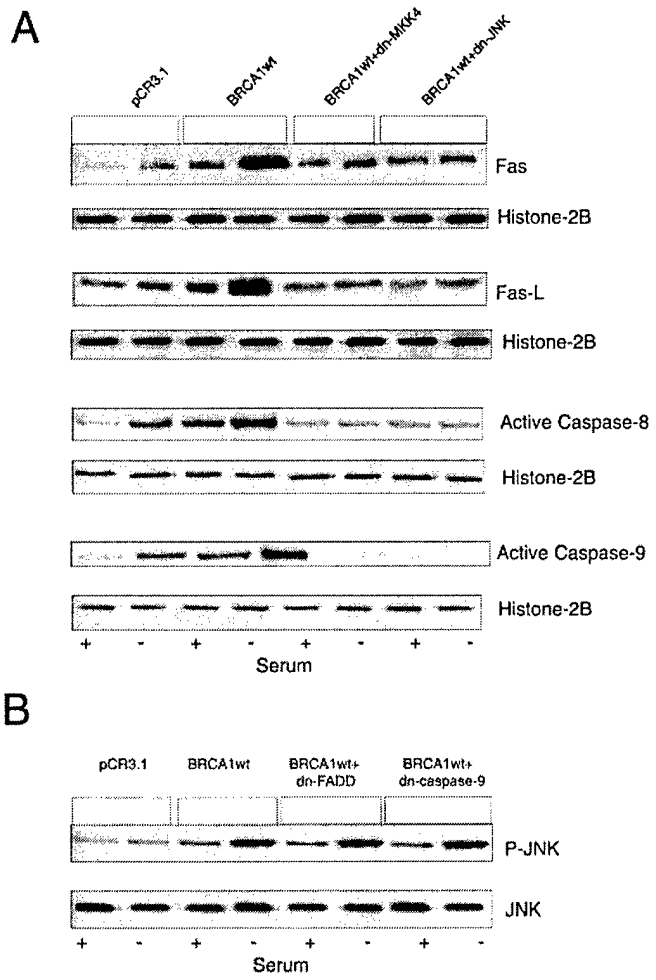

FIG. 6. **The MAPK signaling pathway is upstream of the death receptor and caspase signaling pathway in BRCA1-dependent, serum withdrawal-induced apoptosis.** *A*, inhibition of MKK4 and JNK signaling abrogates Fas and FasL induction and caspase-8 and caspase-9 activation. MCF7 cells were cotransfected with GFP and dnMKK4 or dnJNK along with wild-type BRCA1 or pCR3.1 vector. Cells were sorted for GFP and incubated in the presence and absence of serum for 24 h. Cell lysates were immunoblotted with antibodies against Fas and FasL and caspase-8 and active caspase-9. *B*, inhibition of Fas signaling and caspase-8 function does not affect JNK activation. MCF7 cells co-transfected with GFP and dn-FADD or CrmA, along with BRCA1 wild-type or pCR3.1 vector, were sorted for GFP, and incubated in the presence or absence of serum for 24 h. Immunoblots of lysates with anti-phospho-JNK antibody were used to assess the level of JNK activation.

Serum Starvation-dependent Apoptotic Pathway—To determine whether JNK activation is upstream or downstream of Fas and caspase activation, we assessed the activation of pathway components after inhibition of other components. Co-expression of dn-MKK4 or dn-JNK with wild-type BRCA1 in MCF7 and OV177 cells inhibited induction of Fas and Fas-L as well as activation of caspases-8 and -9, suggesting that the JNK signaling cascade is upstream of death receptor activation and caspase processing (Fig. 6*A*). In contrast, dn-caspase-9 and dn-FADD had no effect on JNK activation (Fig. 6*B*). Thus, the BRCA1-dependent apoptotic pathway that is induced by serum withdrawal appears to signal sequentially from Ras/JNK/Fas/caspase-9.

BRCA1-dependent Apoptosis Is p53-independent—The tumor suppressor protein p53 has been reported to play a critical role in regulating the Fas apoptotic signaling pathway after DNA damage (42–44). To determine whether p53 is involved in the BRCA1-dependent pathway outlined above, we assessed the apoptotic response of cells that lack p53 to serum starvation following transfection with BRCA1. Full-length BRCA1, BRCA1Δ5382-insC, and pCR3.1 were transfected into $p53^{-/-}$ MEF cells or MCF7 cells stably expressing the human papilloma virus (HPV) *E6* gene (MCF7- E6) (45). Immunoblots using the N-terminal BRCA1 antibody showed that both wild-type BRCA1 and the 180-kDa BRCA1Δ5382-insC mutant were expressed in these cells (Fig. 7*A*). Overexpression of full-length BRCA1 in both MEF $p53^{-/-}$ and MCF7 E6 cells induced apoptosis (Fig. 7*B*). Further increases in the levels of apoptosis were observed following serum withdrawal (Fig. 7*B*). As seen in MCF7 and OV177 cells, expression of stoichiometric amounts of BRCA1Δ5382-insC (Fig. 7*A*) abolished all apoptosis associated with endogenous and ectopically expressed BRCA1 in MCF7 E6 and MEF $p53^{-/-}$ cells (Fig. 7*B*). These studies suggest that loss of p53 function has no effect on the BRCA1-dependent response to serum withdrawal.

To determine whether BRCA1-mediated apoptosis occurs through the same pathway as defined above in the absence of functional p53, we examined the effect of Nok2 antibody and expression of dn-FADD on serum withdrawal-induced apoptosis in MCF7 E6 cells (Fig. 7*C*). Both Nok2 and dn-FADD abolished BRCA1-induced apoptosis (Fig. 7*C*). Similar results were observed when these cells were transfected with CrmA and dn-caspase-9 (Fig. 7*C*), suggesting that the entire signal transduction pathway appears to function independently of p53.

DISCUSSION

The results of the present study demonstrate that BRCA1 enhances apoptosis induced by other stimuli in breast and ovarian cancer cell lines; that a truncation mutant lacking the C terminus of BRCA1 suppresses apoptosis, including spontaneous apoptosis, in these cell lines; that BRCA1-dependent apoptosis occurring after serum withdrawal proceeds through a H-Ras/MEKK4/JNK signaling pathway followed by increased expression of Fas and FasL and by activation of caspase-8; and that this Fas-dependent signaling pathway is independent of p53 function. Each of these observations has potentially important implications for current understanding of the role of BRCA1 as a tumor suppressor protein.

A previous study indicated that BRCA1 overexpression can result in induction of high levels of spontaneous apoptosis in U2OS osteosarcoma cells (28). In the present study, overexpression of BRCA1 in breast and ovarian cancer cells was associated with much more modest levels of apoptosis, with 10–12% of the cells undergoing apoptosis in the absence of apoptotic stimuli. The difference between our results and those obtained previously might reflect differences in cell type or in the degree of BRCA1 overexpression. However, the lower levels of apoptosis observed after BRCA1 transfection allowed us to examine the effect of BRCA1 on sensitivity of cells to other apoptotic stimuli. Results of these studies demonstrated that BRCA1 overexpression enhanced the apoptotic response to a variety of stimuli, including withdrawal of serum-derived survival factors, exposure to ionizing radiation, or treatment with the chemotherapeutic agent paclitaxel. These observations suggest that BRCA1 is capable of modulating the apoptotic response to a variety of stimuli.

To further evaluate the effects of BRCA1 on apoptosis, we examined BRCA1 null HCC1937 cells (46). Levels of apoptosis remained low in these cells even after serum starvation, but increased substantially upon ectopic expression of wild-type BRCA1. Conversely, expression of stoichiometric levels of certain BRCA1 truncation mutants was shown to decrease spontaneous and serum withdrawal-induced apoptosis in cells expressing wild-type BRCA1. These observations not only help establish a role for endogenous BRCA1 in the apoptotic re-

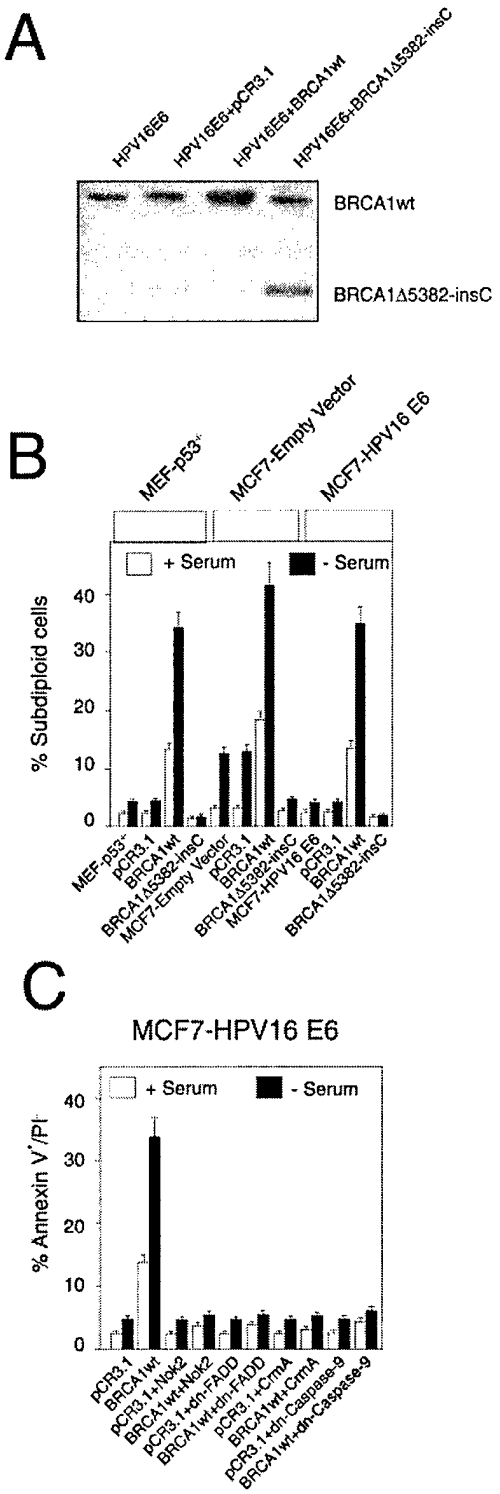

FIG. 7. **BRCA1-dependent apoptosis in serum-deprived breast and ovarian cancer cells is p53 independent.** *A*, immunoblot analysis of BRCA1 expression in MCF7 cells stably expressing human papilloma virus 16 *E6* gene (*HPV16 E6*) following transient transfection with pCR3.1 vector, wild-type BRCA1, and BRCA1Δ5382-insC. Aliquots containing 100 μg of protein were probed with the N-terminal BRCA1 (66036E) antibody. BRCA1 migrated as a 220-kDa band. The BRCA1Δ5382-insC mutant encodes a 180-kDa polypeptide. *B*, BRCA1 enhances apoptosis after serum deprivation in the absence of functional p53. p53 null mouse embryo fibroblasts (MEFs) and MCF7 cells expressing HPV16 E6, or vector control, were transiently transfected with pCR3.1 vector, wild-type BRCA1, or BRCA1Δ5382-insC mutant, along with GFP. The GFP-positive cells were isolated by FACS, incubated in the presence and absence of serum for 24 h, fixed, and stained with PI. The percentage of subdiploid cells is shown. *C*, inhibition of Fas signaling or caspase function ablates BRCA1-dependent apoptosis in MCF7-HPV16 E6 cells. MCF7-HPV16 E6 cells were transiently transfected with pCR3.1 vector or wild-type BRCA1 along with dn-FADD, CrmA, or dn-caspase-9. Cells were also treated with Nok2 and ZB4 and incubated in the presence and absence of serum for 24 h. Apoptosis was assessed after labeling with Annexin V and PI.

sponse, but also suggest that certain BRCA1 truncation mutants can dampen this response in a dominant negative fashion. The BRCA1 mutants used in these studies were truncated before the C-terminal BRCT domains. This deleted region of the BRCA1 protein has been shown to contain two transactivation domains (14, 15, 47) and an RNA helicase binding domain that facilitates interaction with the RNA polymerase II holoenzyme (48). In addition, this region of BRCA1 is known to interact with histone deacetylase (49), the CtIP transcriptional repressor (20, 50), and with p53 (51, 52). Moreover, Abbott and colleagues (53) have shown that the C terminus of BRCA1 is required for transcription-coupled repair and improved cell viability in response to DNA damage. Thus, the mutants that were used in this study were expected to have lost the ability to regulate many BRCA1-associated pathways within the cell. In this study the BRCA1 truncation mutants blocked all apoptosis associated with endogenous or ectopically expressed BRCA1, suggesting that the C terminus of BRCA1 also plays an important role in regulation of apoptosis. Because the C terminus of BRCA1 contains transactivation domains that are involved in transcriptional activation of a number of genes, including $p21^{waf1/cip1}$, *Bax*, *GADD45*, *GADD143*, and IFN-γ, as well as repression of several genes, including *cyclin B1* (19, 22, 26, 28, 51, 54), it is possible that these or other transcriptional targets of BRCA1 might regulate the apoptotic process described in this study.

Harkin and colleagues (28) reported that BRCA1-induced apoptosis in U2OS osteosarcoma cells is associated with JNK activation. However, the apoptotic signaling pathways upstream of JNK in this model system were not reported. Likewise, the signaling pathways upstream of JNK in nerve growth factor withdrawal-induced apoptosis in neuronal PC12 (34) and in detachment-associated apoptosis (anoikis) in various cell types (55, 56) have not been well defined. In the present study, we investigated the signal transduction pathway that was activated in a BRCA1-dependent manner by serum withdrawal. Results of this analysis identified a pathway involving activation of MEKK4 and JNK by phosphorylation. This pathway was inhibited by Ras N17, dn-MKK4, dn-MEKK4, and dn-JNK, pointing to a pathway that involves sequential signaling from H-Ras to MEKK4, MKK4, and JNK. Although the apparent involvement of H-Ras in this pathway was somewhat unexpected, a number of studies have recently shown that Ras proteins can regulate apoptotic responses in a cell type- and stimulus-dependent fashion (33). In particular, it has been shown that Ras can induce apoptosis by binding and activating MEKK1 (31). Other studies have refined this model by demonstrating that Ras induces apoptosis through a Rac1- and p21-activated kinase-dependent pathway (57), and through a Ras/Rac1/CDC42/MLK3 (mixed lineage kinase 3)/MEKK pathway (58). Most recently, the Ras-associated apoptotic pathway has been shown to signal through JNK in a p53-independent manner (59) similar to the pathway identified above. In the present study we did not attempt to define the specific signaling pathway upstream of H-Ras, nor did we determine how BRCA1 is modulating signaling through this pathway. These are areas for future investigation.

Although BRCA1 was shown to activate JNK in U2OS cells (28), the pathways downstream of JNK in BRCA1-dependent apoptosis were not reported. Previous studies have raised the possibility that JNK can function as a downstream potentiator

of Fas-induced apoptosis through caspase and DAXX activation (60, 61), or as an inhibitor of tumor necrosis factor-induced apoptosis as seen in lymphocytes from JNKK1 and traf2 null animals (62, 63). Our results indicated a different role for JNK. After serum withdrawal, we observed BRCA1-dependent up-regulation of both FasL and Fas. The ability of dn-JNK to abrogate FasL and Fas induction placed JNK upstream of FasL and Fas in the apoptotic signaling pathway. These results are consistent with recent reports implicating JNK-dependent activation of the Fas/FasL pathway in nerve growth factor withdrawal-induced apoptosis in neuronal PC12 cells (34), stress-induced apoptosis in Jurkat cells (35), and cell detachment-associated apoptosis (anoikis) in various cell types (55, 56). These results also extend these previous studies by demonstrating a role for BRCA1 in modulating signaling through the JNK/FasL/Fas pathway.

The ability of the blocking antibodies ZB4 and Nok2 to abrogate BRCA1-dependent serum withdrawal-induced apoptosis provided strong evidence that the up-regulation of FasL and its interaction with Fas are critical to this death process. Consistent with these results, abrogation of FADD signaling and inhibition of caspase-8 (through expression of CrmA) also inhibited the apoptotic response to serum withdrawal. In addition, expression of dn-caspase-9 inhibited this apoptotic pathway, suggesting that activation of caspase-8 results in activation of caspase-9 through a mitochondrial pathway, as has been suggested for "type II" cells (37, 39, 64). Furthermore, because this apoptotic pathway appears to retain activity in the absence of caspase-3, which is known to be down-regulated in MCF7 cells (37), the suggestion is that the caspase-3 effector is not required for this effect. Thus, it is likely that other effector caspases, such as caspase-7, may also mediate processing of multiple cellular targets as part of the BRCA1-dependent apoptotic pathway.

A number of studies have reported that FasL/Fas signaling after DNA damage requires the action of the tumor suppressor protein p53 (42–44). In the present study, we determined whether loss of p53 affected the BRCA1-dependent serum withdrawal-induced Fas/FasL pathway. The signal transduction pathway defined above was activated in a BRCA1-dependent manner in two cell models lacking p53 function. These results suggest that activation of the Fas/FasL after growth factor withdrawal proceeds by a pathway that is distinct from DNA damage-induced Fas/FasL activation.

In summary, we have established that the BRCA1 tumor suppressor functions as a regulator of apoptosis in response to serum deprivation and a number of other apoptotic stimuli. We have delineated a BRCA1-dependent, serum withdrawal-induced apoptotic pathway that sequentially involves H-Ras, MEKK4, and JNK followed by induction of FasL and activation of FADD- and caspase-9-dependent signaling. In addition, we have demonstrated that activation of this pathway is p53-independent and we have identified novel dominant negative activity of BRCA1 truncation mutants. These results provide an improved understanding of the tumor suppressor function of BRCA1 and suggest that the multifunctional BRCA1 protein coordinately regulates apoptotic events in addition to its effects on the DNA damage response, cell cycle progression, and transcription.

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